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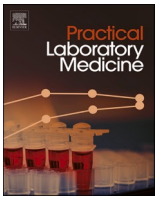

Application of a clamshell isothermal nucleic acid amplification analyzer in the detection of lower respiratory tract bacteria

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ABSTRACT

Objectives: The clamshell isothermal nucleic acid amplification analyzer RTisochip-S, a next-generation instrument featuring improved structural design, enhanced functional integration, reduced cost, and increased portability, was assessed for its suitability in clinical respiratory pathogens detection.
Methods: The certificated detection kit for lower respiratory tract bacteria (LRTB-kit) was applied to evaluate the performance of RTisochip-S via sensitivity, specificity, and repeatability analysis. The clinical specimens, including 51 sputum specimens and 10 bronchoalveolar lavage fluid specimens, were simultaneously detected on both RTisochip-S and a certificated reference instrument (RTisochip-A) to assess the consistency.
Results: The results indicated that RTisochip-S fulfills the sensitivity, specificity, and repeatability requirements of the LRTB-Kit, and the results of clinical specimens on the two instruments were consistent.
Conclusions: RTisochip-S is satisfying the clinical detection of respiratory pathogens while enhancing portability and compactness, making it more well-suited for point-of-care testing (POCT) applications.

1. Introduction

The detection of nucleic acids is extensively employed in the diagnosis, prevention, and control of infectious diseases, as well as in early adjunctive diagnosis and monitoring of tumors, genetic disease identification, prenatal screening, etc [1–4]. In recent years, there has been a persistent high frequency of infectious disease outbreaks, particularly respiratory tract infections. According to the World Health Organization (WHO), lower respiratory tract infection was the fourth leading cause of death worldwide in 2019 [5]. The coronavirus disease 2019 (COVID-2019) outbreak has presented unprecedented challenges to the timely and accurate screening of cases, underscoring the imperative for point-of-care testing (POCT) protocols in nucleic acid testing. PCR is widely regarded as the gold

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standard for nucleic acid detection [6]; however, its application in POCT is limited due to the requirement of thermal cycling [7]. The emerging isothermal amplification technology (ITA), in contrast, utilizes DNA polymerase with chain replacement activity to rapidly amplify nucleic acids at a constant temperature, thereby simplifying the temperature cycling required for amplification. This reduces the complexity of detection instruments and makes ITA more suitable for POCT [8,9]. The current ITA primarily encompasses loop-mediated isothermal amplification (LAMP), nuclear acid sequence-based amplification (NASBA), rolling circle amplification (RCA), recombinase polymerase amplification, and others [9]. Recently, isothermal amplification techniques such as LAMP, NASBA, and RCA have been integrated into microfluidic devices [10–15], cross-interface emulsification [16,17], CRISPR/Cas gene editing [18, 19], and other technologies. The field of ITA is progressively advancing towards automatic integration and information processing while achieving miniaturization and enhancing overall performance.

RTisochip-A, the certificated reference instrument used in this study, is a compact nucleic acid amplification and analysis instrument measuring 365 mm × 300 mm × 205 mm and weighing 8 kg. An integrated microfluidic chip, equipped with the RTisochip-A, enables precise nucleic acid detection on a micro-scale using only 1.45 μL per reaction, thereby significantly reducing reagent consumption and overall detection costs [20]. Moreover, this versatile instrument and microfluidic chip enable the parallel qualitative identification of up to 24 detection indicators simultaneously without the risk of cross-contamination, while exhibiting extremely high sensitivity with a limit of detection ranging from 10 to 10^3 genome copies [20]. They effectively cater to the requirements for nucleic acid detection in sputum and alveolar lavage fluid specimens by offering rapid results with high sensitivity and specificity [20–23]. However, the portability of RTisochip-A is limited by its requirement for a separate centrifugal device. To enhance the applicability of POCT, we have refined RTisochip-A into a next-generation instrument, namely RTisochip-S. This advancement encompasses structural design optimization, refinement of the detection module, improvement in fluorescence acquisition elements, enhancement of functional integration, as well as reduction in volume and weight. We applied a domestic confocal optical path structure of PIN tube to replace the costly imported photomultiplier tube from Japan used in RTisochip-A for fluorescence signal collection. This modification enables efficient acquisition of fluorescence signals while reducing costs. Additionally, a manual clamshell was used to replace the mechanical access bin and a self-locking seal was employed to substitute a mechanical seal. These modifications significantly reduce the number of mechanical moving parts in the instrument, resulting in a decrease in production costs, improved convenience for subsequent installation and debugging, and achievement of equipment miniaturization, with a 76.90 % reduction in volume and a 62.5 % reduction in weight compared to RTisochip-A. Furthermore, RTisochip-S is equipped with an integrated drive structure, enabling simultaneous high-speed (≥6000 rpm) rotation for sampling and low-speed (≤2 rpm) rotation for nucleic acid amplification and detection, thus achieving the integration of centrifugation and detection. This eliminates the need for an additional centrifuge, as required by RTisochip-A. In summary, the RTisochip-S is a lighter (with dimensions of 260 mm by 189 mm by 105.5 mm and weighing only 3 kg) and more cost-effective instrument, demonstrating significant advantages for POCT.

This study assessed the clinical test performance of the next-generation instrument RTisochip-S using the certificated detection kit. The sensitivity, specificity, and repeatability of the kit were verified on RTisochip-S. Simultaneously, the clinical specimens were detected on both RTisochip-S and a certificated reference instrument (RTisochip-A) to assess the instrument's consistency.

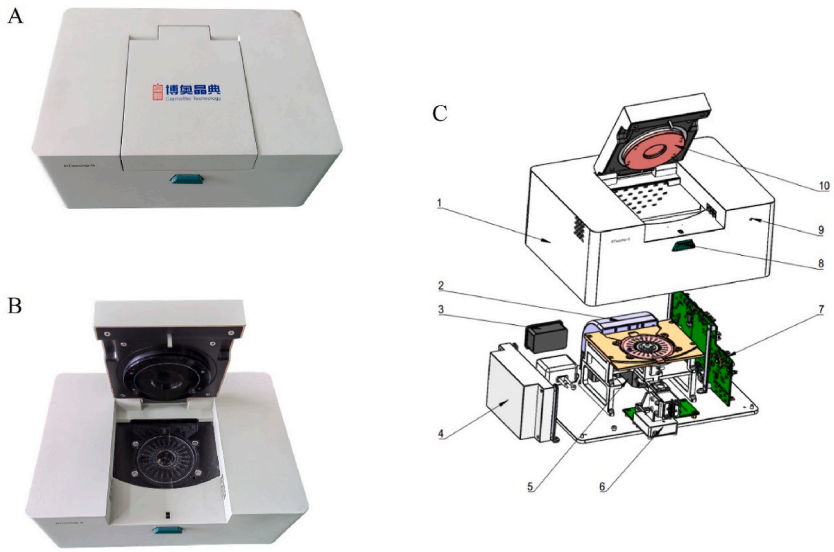

Fig. 1. Clamshell isothermal nucleic acid amplification analyzer RTisochip-S. (A) Diagram illustrating instrument appearance. (B) Diagram depicting the opening of instrument cover. (C) Exploded diagram showcasing instrument structure. In Figure C, the labels indicate: 1. Shell; 2. Heat dissipation component; 3. Power socket; 4. Switching power supply; 5. Power module; 6. Optical path component; 7. Main control board; 8. Locking mechanism component; 9. Status indicator light; 10. Floating top cover component.

2. Material and methods

2.1. Instruments

The nucleic acid extractor, microfluidic chip centrifuge, and isothermal amplification microfluidic chip nucleic acid analyzer (RTisochip-A and RTisochip-S) were all obtained from CapitalBio Technology (Chengdu) (Sichuan, China). The appearance diagram and explosion diagram of RTisochip-S instrument are depicted in Fig. 1. The instrument comprises a power supply module, a temperature control module, a detection module, a data connection module, and a software module.

2.2. Reagents and consumables

The bacterial genomic DNA extraction kit (glass bead method) and the lower respiratory tract bacteria nucleic acid detection kit (isothermal amplification chip method), hereinafter referred to as LRTB-Kit were purchased from CapitalBio Technology (Chengdu). The LRTB-Kit contains multiple quality control principles to ensure the reliability of the detection results. The chip of the kit contains nine "no template" (negative) control (NC), a positive control (PC) targeting *Escherichia coli*-specific sequence, a positive external control (PEC) targeting yeast-specific sequence, and an internal control (IC) targeting the RNase P gene.

Additionally, the human genomic DNA reference material with a concentration of 50 ng/μL, was also obtained from CapitalBio Technology (Chengdu), along with the negative and positive reference materials for lower respiratory tract bacteria (LRTB). The eight positive reference materials, each with a concentration of 1×10^5 copies/μL, comprised the complete sequence of *Streptococcus pneumonia* (Spn), *Staphylococcus aureus* (Sau), methicillin-resistant *Staphylococcus* aureus (MRSA), *Klebsiella pneumonia* (Kpn), *Pseudomonas aeruginosa* (Pae), *Acinetobacter baumannii* (Aba), *Stenotrophomonas maltophilia* (Sma) and *Haemophilus influenzae* (Hin). The five negative reference materials, also with a concentration of 1×10^5 copies/μL, included the complete sequence of *Proteus vulgaris*, *Enterococcus faecium*, *Streptococcus mutans*, *Citrobacter freundii*, and *Acinetobacter lwoffii*. The LRTB-Kit was employed to identify eight clinically prevalent pathogens in the lower respiratory tract specimens, namely Spn, Sau, MRSA, Kpn, Pae, Aba, Sma, and Hin.

2.3. Clinical specimens and ethics statement

Between May 4 and June 8, 2023, we randomly selected 51 sputum specimens and 10 bronchoalveolar lavage fluid specimens from the remaining specimens tested at Chengdu CapitalBio Medical Laboratory. The study was approved by the ethics review committees of Fujian CapitalBio Medical Laboratory, China (K2024-01-001), in accordance with all relevant guidelines and regulations. The patient data were fully anonymized in our study, and the ethics review committee waived the requirement for informed consent.

2.4. Nucleic acid extraction (glass bead method)

The sputum specimens (or bronchoalveolar lavage fluid specimens) were mixed with an equal volume of 4 % NaOH and incubated at 37 °C for 30 min to induce liquefaction. The supernatant was carefully decanted after centrifugation at 12,000 rpm for 5 min. The resulting precipitate was resuspended in a washing solution, followed by another centrifugation step (12,000 rpm, 5 min), and subsequent removal of the supernatant to obtain the bacteria. 100 μL nucleic acid extract was added to the collected bacteria, thoroughly shaken, and mixed. Subsequently, the mixture was heated in a metal bath at 95 °C for 5 min and then subjected to centrifugation at 12,000 rpm for another 5 min. Finally, the supernatant containing extracted nucleic acid was transferred into a clean centrifuge tube for immediate use or stored at −20 °C for no more than two months.

2.5. Sample addition and nucleic acid amplification

The quality of the experimental environment and personnel operation was monitored by employing a PC material obtained from the LRTB-Kit and a NC material (DNase-free water) to react on separate chips, respectively, after each kit opening. The subsequent experiments can only be conducted if the controls are deemed valid.

The isothermal amplification reagent (20 μL) was mixed with the tested nucleic acid sample (or control substance) (34.5 μL) to prepare the isothermal amplification reaction system. Subsequently, 50 μL of the amplification reaction system was drawn into the main channel of the chip through sample inlet 1 until it reached full capacity. After covering the sealing membrane on the inlet and exit sample ports, centrifugation of the chip was performed at 6000 rpm for 30 s. Finally, the chip was placed in an isothermal nucleic acid amplification analyzer for subsequent nucleic acid detection using an amplification program consisting of incubation at 37 °C for 3 min followed by incubation at 65 °C for 47 min.

2.6. Result interpretation

Nine NC, a PEC, and an IC were used in each detection run to ensure the reliability of the results. NC enables monitoring for potential contamination during the experimental process. PEC allows monitoring for the proper experimental process. And IC is utilized to monitor sample collection, handling, and experimental process to avoid false-negative results. The assessment of sample test results should be performed after the NC, PEC, and IC have been examined and determined to be valid and acceptable. If any of the controls are not valid, the sample results cannot be interpreted.

After detection, the software employed the maximum second derivative method in conjunction with other algorithms to calculate the first inflection point of the "S-type" amplification curve, thereby determining the onset of rapid amplification. The Tp value was defined as the difference between this inflection point and the origin, and the result interpretation was based on a combination of the Tp value and positive threshold. Notably, each index had a distinct positive threshold (Table 1). A value exceeding this threshold was denoted as "-1", indicating absence of pathogen nucleic acid; conversely, values equal to or below it were considered presence of pathogen nucleic acid.

2.7. Sensitivity test

The eight positive reference materials (P1–P3, P5–P9) of LRTB were diluted to detection limit concentration (5×10^2 copies/μL) using 1 × TE solution and designed as $S1_{-1}$-$S3_{-1}$, $S5_{-1}$-$S9_{-1}$. Subsequently, the sensitivity was assessed utilizing the LRTB-Kit. The test results were considered satisfactory if the positive coincidence rate was ≥95 %.

2.8. Specificity test

The human genomic DNA reference material (N1) was diluted to a concentration of 10 ng/μL, serving as the positive internal control. Additionally, the negative reference materials (N2–N6) with a concentration of 1×10^5 copies/μL were utilized, along with the NC and PC materials. These eight samples were employed to assess the specificity of the LRTB-Kit for LRTB. To meet the requirements for specificity, all eight test results for LRTB should be negative, with a negative coincidence rate ≥95 %.

2.9. Repeatability test

The eight positive reference materials of LRTB (P1–P3, P5–P9) were mixed in the same volume and diluted with 1 × TE solution to a final concentration of 8×10^3 copies/μL for each pathogen (R1). R1 underwent five repeated experiments using RTisochip-S instrument with the LRTB-Kit. The mean, standard deviation (SD), and coefficient of variation (CV) were calculated for each pathogen based on the results. Reproducibility was considered acceptable if all eight pathogens tested positive and CV ≤ 10 %.

2.10. Evaluation and verification of clinical specimens

Sputum specimens ($n = 51$) and bronchoalveolar lavage fluid specimens ($n = 10$), obtained from Chengdu CapitalBio Medical Laboratory, were analyzed for respiratory pathogen nucleic acid detection using the RTisochip-A and RTisochip-S instrument, respectively. The rates of pathogen detection consistency were separately calculated for two different specimen types on two instruments, and their Tp values were compared. The consistency of the detection results was assessed by calculating the Cohen's kappa coefficient (κ), where a κ value less than 0.40 indicates poor agreement, $0.4 \leq \kappa < 0.75$ indicates moderate agreement, and $\kappa \geq 0.75$ indicates good agreement. Mean, SD, and CV were utilized to analyze and compare Tp values.

2.11. Statistical analysis

The statistical analysis was conducted using SPSS software. The measurement data were presented as mean and SD, while the Kappa test was employed to assess the consistency of qualitative data.

3. Results

3.1. Sensitivity evaluation

The sensitivity of the LRTB-Kit with the RTisochip-S instrument was evaluated by diluting eight positive reference materials ($S1_{-1}$-$S3_{-1}$, $S5_{-1}$-$S9_{-1}$). The results demonstrated that all samples tested positive for the corresponding LRTB, indicating a sensitivity of 100 %

Table 1
Sensitivity evaluation results.

Numbering of the positive reference materials	Detection indicators and their corresponding Tp value thresholds	The Tp value of the detection result	Interpretation of results
$S1_{-1}$	Spn (≤30)	17.04	Spn positive
$S2_{-1}$	Sau (≤34)	15.57	Sau positive
$S3_{-1}$	Sau (≤34)/MRSA (≤22)	15.09/11.17	Sau and MRSA positive
$S5_{-1}$	Kpn (≤29)	20.22	Kpn positive
$S6_{-1}$	Pae (≤36)	18.22	Pae positive
$S7_{-1}$	Aba (≤36)	14.78	Aba positive
$S8_{-1}$	Sma (≤36)	20.36	Sma positive
$S9_{-1}$	Hin (≤36)	17.89	Hin positive

(Table 1). Thus, the RTisochip-S instrument fulfills the sensitivity requirements of the LRTB-Kit for LRTB.

3.2. Specificity evaluation

The results showed the LRTB-Kit had no cross-activity with human genomic DNA, *Proteus vulgaris*, *Enterococcus faecium*, *Streptococcus mutans*, *Citrobacter freundii*, and *Acinetobacter lwoffii*, while the primer/probe group exhibited no self-reactivity. Therefore, RTisochip-S meets the specificity requirements of the LRTB-Kit for LRTB.

3.3. Repeatability evaluation

The statistical results of repeatability evaluation are presented in Table 2. The detection results of all eight respiratory pathogen indicators in the five repeated experiments were positive, with CV $\leq$ 10 % for all indicators. Thus, RTisochip-S was demonstrated to satisfy the reproducibility requirements of the LRTB-Kit.

3.4. Validation results of clinical specimens

The consistency of instrument results was evaluated by testing clinical specimens on both RTisochip-S and RTisochip-A. The results revealed the detection of 19 cases of Spn, 13 cases of Sau, 20 cases of MRSA, 1 case of Kpn, 1 case of Aba, 27 cases of Hin, and 11 cases of negative out of a total of 51 sputum specimens. There was complete concordance between the results obtained using the two instruments.

4. Discussion

The RTisochip-S instrument was subjected to preclinical testing by evaluating the sensitivity, specificity, and repeatability of the LRTB-Kit for LRTB, ensuring its compliance with the LRTB-Kit's requirements. Subsequently, the clinical specimens were analyzed using both RTisochip-S and the·certificated reference instrument RTisochip-A to ensure consistency of results across different instruments. The LRTB-Kit exhibited a 100 % detection rate for the targeted eight LRTBs without any cross-reaction with human genomic DNA or five negative reference materials, indicating a high sensitivity and specificity. Additionally, satisfactory repeatability was confirmed with a CV $\leq$ 10 % for each pathogen. Moreover, Table 3 presents the mean, SD, and CV for Tp values of Spn, Sau, MRSA, and Hin in the test results from both instruments, considering only these with more than 10 positive detections. The results indicated that the mean value obtained with RTisochip-S is higher than that with RTisochip-A; however, this disparity didn't impact the interpretation of qualitative results. This could potentially be attributed to the implementation of a confocal optical path structure based on PIN tubes in RTisochip-S, as opposed to utilizing imported fluorescence photomultiplier tubes in RTisochip-A. Importantly, there was complete concordance between the results obtained using RTisochip-S and RTisochip-A. The test results of bronchoalveolar lavage fluid specimens from both instruments were also complete consistency; however, due to the limited number of specimens, it was unable to assess the difference in Tp values detected by both instruments. This should be further evaluated by increasing the sample size. In summary, RTisochip-S demonstrates the capability to fulfill the sensitivity, specificity, and repeatability requirements of the LRTB-Kit for LRTB while catering to the demands of clinical specimen detection.

The next-generation instrument RTisochip-S offers crucial support and assurance for POCT in detecting pathogenic microorganisms, as well as providing novel sights for future instrument design improvements. In the future, to enhance overall efficiency while minimizing aerosol contamination, we will optimize the optical path structure based on RTisochip-S and further integrate functions such as specimen lysis, nucleic acid extraction, amplification, and detection to achieve a fully enclosed and automated "specimen in - result out" model. Furthermore, we will develop RTisochip-S-based detection kits tailored to diverse diagnostic requirements, encompassing respiratory viruses (such as SARS-CoV-2 and influenza virus), mycoplasma, and other pathogens.

Table 2
Statistics of repeatability evaluation results.

Detection indicators	Number of positives	Tp value		
		Mean	SD	CV (%)
Spn	5	16.60	1.31	7.91
Sau	5	14.59	0.83	5.71
MRSA	5	12.18	0.48	3.96
Kpn	5	12.69	0.67	5.29
Pae	5	16.25	1.35	8.31
Aba	5	12.29	0.45	3.69
Sma	5	13.30	1.26	9.48
Hin	5	14.82	0.98	6.65

Table 3
Statistical results of sputum specimens determined by RTisochip-A and RTisochip-S instruments.

Detection indicators	RTisochip-A			RTisochip-S			Deviation of RTisochip-S compared to RTisochip-A (%)
	Mean	SD	CV (%)	Mean	SD	CV (%)	
Spn	17.05	2.86	16.79	18.91	3.96	20.94	10.90
Sau	20.76	5.01	24.15	23.09	8.09	35.02	11.22
MRSA	18.36	6.17	33.63	18.6	5.61	30.15	1.31
Hin	23.21	6.78	29.22	27.34	7.98	29.18	17.79

5. Conclusion

RTisochip-S is satisfying the clinical detection of respiratory pathogens while enhancing portability and compactness, making it more well-suited for POCT applications.

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Compliance statement

The composition and operation of the Ethics committee adhere to pertinent laws and regulations.

CRediT authorship contribution statement

Guanbin Zhang: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Xiaoying Lin:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Wenkun Mu:** Validation, Supervision, Resources, Investigation. **Jun Luo:** Validation, Resources, Investigation. **Yiyuan Xu:** Writing – review & editing, Methodology, Formal analysis. **Chicheng Song:** Validation, Supervision, Investigation. **Jiang Li:** Visualization, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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