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Rapid Detection of *Enterobacter cloacae* With a Visualized Isothermal Recombinase Polymerase Amplification Assay

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Abstract

Enterobacter cloacae exhibits strong adhesion and invasion properties that contribute its ability to infect the host; it is considered an important opportunistic pathogen throughout the world. To control the spread of *E. cloacae*, simple, rapid, and accurate detection methods are required. Current methods suffer from various shortcomings and do not meet the demand for on-site quickly detection. Using recombinase polymerase amplification combined with lateral flow strip (RPA-LFS), an isothermal detection method was developed to target the outer membrane protein X (*omp*X) gene of *E. cloacae.* This reaction can be performed in 30 min at 37 °C. Limit of detection of 10 CFU/reaction was equivalent to that of the qPCR method. The detection accuracy of clinical samples was also equal to that of the qPCR method. In this study, we developed the RPA-LFS assay, which is simple, rapid, accurate, and does not require a laboratory facility. This assay may prove useful for detecting *E. cloacae* on-site.

Introduction

Enterobacter cloacae (*E. cloacae*) belongs to the *Enterobacter* genus and is a kind of Gram-negative bacterium [1]. It has been widely found in soil, plants, and various aquatic environments [2]. It has strong adhesion and invasion properties that contribute to its ability to infect the host [3]. *E. cloacae* is an important opportunistic pathogen and the leading cause of nosocomial infections worldwide [4, 5]. It can cause septicemia, meningitis, endocarditis, septic arthritis, and bone marrow. It is also possible for inflammation to cause infections of the lower respiratory tract, skin, soft tissues, urinary tract, abdominal cavity, and eyes [4, 6]. Recently, there have been many reports of *E. cloacae* infections in veterinary clinics. *E. cloacae* can also cause diseases in aquatic animals and cause a large number of deaths [7]. In remote areas, it can result in the deaths of many aquatic animals on farms, due to a failure to detect and treat in time.

Now, with the overuse of antibiotics, *E. cloacae* has a high prevalence of multi-drug resistance [8]. This brings several challenges to clinical treatment and infection control [9, 10]. To deal with the global epidemic of *E. cloacae*, monitoring and early identification is important for human life, farm animals, and aquaculture.

The diagnosis of pathogens is essential for the prevention and treatment of diseases for both patients and hospitals [11]. Traditional culture methods can identify pathogen accurately, but are a lengthy process. Recently, many functional enzymes have been used to establish conventional methods of biochemical identification of clinical pathogens, such as polymerase chain reaction (PCR)-based, quantitative PCR (qPCR)-based, biochemical analysis, conventional culture procedure, and immunology-based diagnosis tests [12, 13–15]. Although many useful detection methods have been developed, they all suffer from a variety of drawbacks. It is possible to perform most of these methods in laboratories with the proper equipment, but it is often necessary to detect

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pathogens in peoples' houses, poorly equipped hospitals, and farms; in these cases, it can be difficult to detect pathogens in a short period of time [16]. It is particularly important to establish a relatively simple and straightforward method as there is often a lack of trained personnel. For this reason, isothermal amplification methods have been established (such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA)) [17, 18].

Recombinase polymerase amplification (RPA), first reported in 2006 [19], it amplifies nucleic acids by using recombinases (UvsX and UvsY), single-stranded binding proteins (gp32), and strand-displacing DNA polymerases (BSU). RPA does not require a stringent incubation temperature; exponential amplification can be performed at 37 °C rather than thermocycling between 55 °C and 95 °C. It also only requires 30 min or less to complete the reaction. RPA amplification products can be detected by gel electrophoresis, real-time fluorescence, and lateral flow strips (LFS), and other methods [17, 20, 21]. Of these detection methods, as detection results can be analyzed with the naked eye, the lateral flow strip is suitable for simple testing and do not require complex instruments and trained personnel.

Materials and Methods

Collection of Samples and DNA Extraction

Reference strains of *Enterobacter cloacae*, *Candida parapsilosis, Candida tropicalis*, *Candida albicans*, and *Acinetobacter baumannii* were obtained from American Type Culture Collection (Manassas, VA, USA). In addition, sputum isolated strains of *E. cloacae* and other infectious pathogens were provided by The Second People's Hospital of Lianyungang (Lianyungang, China). Clinical samples were collected from patients. Strain information is provided in Table 1. All strains have been confirmed by 16S rRNA sequencing [22]. The strains used in this study were cultured in Luria–Bertani (LB) broth at 37 °C for 200 rpm. Cultures of 10^7 colony-forming units per microliter (CFU/μL) were tenfold diluted from 10^6–10^0 CFU/μL as templates. All templates were boiled at 100 °C for 10 min, and RPA-LFS was detected using a template of 1 μL.

Table 1 Bacterial strains used in this study

Species	Source	Strain designation
E. cloacae	Reference strain	ATCC 13,047
E. cloacae	Reference strain	ATCC 23,355
E. cloacae	Reference strain	ATCC 35,030
E. cloacae	Reference strain	ATCC 49,141
E. cloacae	Sputum isolated strain	#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13 #14 #15 #16
C. parapsilosis	Reference strain	ATCC 22,019
C. tropicalis	Reference strain	ATCC 20,962
C. albicans	Reference strain	ATCC 10,231
C. auris	Sputum isolated strain	N/A
C. dubliniensis	Sputum isolated strain	N/A
C. glabrata	Sputum isolated strain	N/A
C. neoformans	Reference strain	ATCC 14,116
A. fumigatus	Sputum isolated strain	N/A
E. faecium	Sputum isolated strain	N/A
E. coli O157	Sputum isolated strain	N/A
P. aeruginosa	Sputum isolated strain	N/A
S. aureus	Sputum isolated strain	N/A
S. capitis	Sputum isolated strain	N/A
S. epidermidis	Sputum isolated strain	N/A
S. haemolyticus	Sputum isolated strain	N/A
S. hominis	Sputum isolated strain	N/A
S. saprophyticus	Sputum isolated strain	N/A
S. warneri	Sputum isolated strain	N/A
S. maltophilia	Sputum isolated strain	N/A
S. pneumonia	Sputum isolated strain	N/A
V. streptococci	Sputum isolated strain	N/A
K. pneumoniae	Sputum isolated strain	N/A
A. baumannii	Reference strain	ATCC 19,606
A. calcoaceticus	Sputum isolated strain	N/A
A. lwoffii	Sputum isolatedstrain	N/A
A. haemolyticus	Sputum isolated strain	N/A
A. junii	Sputum isolated strain	N/A

ATCC American Type Culture Collection (Manassas, VA, USA)

Design of Primers and Probes

An RPA-LFS assay was developed using primers and probes targeted at the sequence of the *omp*X gene (GenBank accession number CP009756.1). The FASTA sequence of the fragment was input into the NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and primer BLAST was performed with the following criteria: product size minimum was 100 and maximum 300; primer size minimum was 30 and maximum 33; primer GC content minimum was 20 and maximum 80.

In the RPA reaction, a probe can increase amplification specificity and reduce primer-dependent artifacts [19]. A spacer C3 group (SpC3) was located at the 3 end of the probe, preventing strand extension, and a tetrahydrofuran group (THF) at the middle (position 32) made the probe function. For the 3 end of the probe to be released from SpC3 blocking, it was necessary for the bases flanking the THF site to pair well with the template. For probe design, the forward primer previously screened was used, and new

forward primers for the 5′ end of the probe. The probe was designed using Primer Premier 5 software. Key parameters were as follows: size of probe, 45–48 bp; melting temperature (*Tm*), 50–100 ˚C; GC content, 20–70%. Other parameters were set as default. Additionally, if the probe and primers matched three times consecutively, to avoid false-positive results, primers and probes were mutated. The primers and probes used in this study are listed in Table 2.

RPA-LFS Procedure

For RPA-LFS detection, the primers and templates mentioned above were used. The Twist Amp® DNA Amplification Kit (TwistDx Ltd., Maidenhead, UK) should be used according to the manufacturer's instructions. In each 50 µL reaction mixture, the following components were included: 2.1 µL of each primer (10 µM), 0.6 µL of probe, and 1 µL of template. Following a few minutes of centrifugation, the reaction was conducted at 37 °C for 30 min. Ten microliters of the RPA product were then spotted on the LFS (Ustar Biotechnologies Ltd., Hangzhou, China). The LFS was composed of a sample pad, conjugate pad (soaked with mouse-originated AuNP-tagged anti-FITC antibody), test line (coated with streptavidin), control line (coated with anti-mouse antibody), and absorbent pad that lined up through the solvent migration route. Test and control lines were visualized after 5 min of immersion in 100 µL of the solvent with the LFS stick containing the RPA product.

The qPCR Procedure

The qPCR detection of *E. cloacae* was performed as reported previously [23]. A pair of specific primers (qPCR-F and qPCR-R) targeting the *omp*X gene of *E. cloacae* was used. The reaction program was set as follows: denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 3 s, and annealing and extension at 60 °C for 20 s on the StepOne™ real-time PCR System from Applied Biosystems (CA, USA).

Results

Design and Screening of RPA Primers and Probe for *E. cloacae* Detection

The gene *omp*X was selected as the target gene for *E. cloacae* detection. RPA detection indicated no nonspecific bands, and the size of the products was consistent with the expected size. The two sets of primer pairs demonstrated good ability to detect *E. cloacae* (Fig. 1a). To avoid primer dimers, the primer–probe set EC-F1/R1 was selected for further screening. RPA-LFS system must be able to incorporate the probe without excessive amplification, the forward primer (EC-F1) was changed to the probe, and possible mismatches with the reverse primer (EC-R1B) was analyzed (Fig. 1b). There can be a few mismatches between primers and probes when using RPA-LFS (Wu et al., 2020). To reduce the possibility of nonspecific amplification, match bases of the reverse primer and probe were changed and marked in red (Table 2). F4/P/

Table2 Primers and probes

Primers/Probes	Primer sequences	Size (bp)	Reaction name
EC-F1	CTACGGTGTTGTAGGTGTTGGCTACGGTAAA	31	RPA
EC-R1	GAGAAGTCCAGAGCAACGTCTTCGATTGGGT	31	
EC-F2	GCACTGCAAGCAACAGCGACTACGGCTTCTC	31	
EC-R2	GTAACCTACGCCCGCGATCCAGGTGCCAACG	31	
EC-P	FITC-CTACGGTGTAGTAGGTGATGGCTACGGTAA A[THF]TCCTGCAGACTGTAA-C3 spacer	46	RPA-LFS
EC-R1B	Biotin-GAGAATTCCGGAGCAACCACTTCGATTGGGT	31	
EC-F3	TTACCGTATCAACGACTGGGCAAGCATCTA	30	
EC-F4	ACTGGGCGTGATCGGTTCTTTCACCTACAC	30	
EC-F5	GGTTTCAACCTGAAGTATCGTTACGAGCAG	30	
EC-F6	TGAAGTATCGTTACGAGCAGGATAACAACCC	30	
EC-F7	AAGTATCGTTACGAGCAGGATAACAACCCA	30	
qPCR-F	TTCCAGCAGACCGAAAACCA	20	qPCR
qPCR-R	AGCAACGTCTTCGATTGGGT	20	

F forward primer; *R* reverse primer; *P* probe

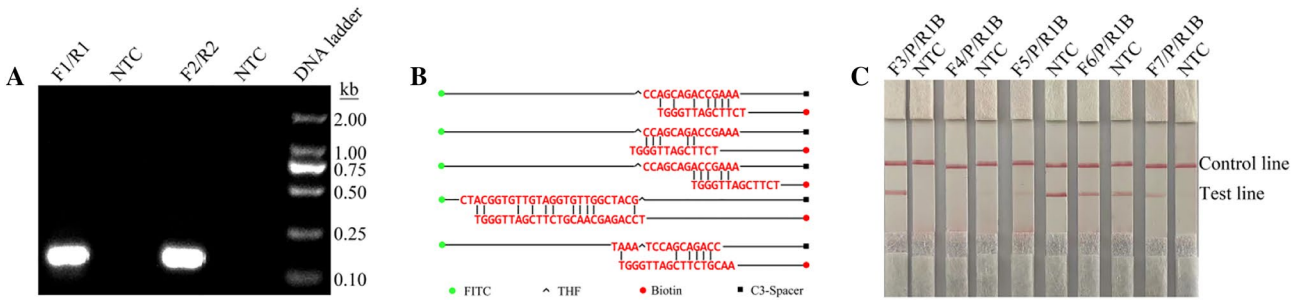

Fig. 1 Primer and probe amplification. **a** Agarose gel showing primer pairs that target the *omp*X gene and their recombinase polymerase amplification (RPA) results. On each lane, primer pairs are indicated. Three independently conducted experiments are represented in the image. **b** Primer Premier 5 software was used to analyze and modify reverse primer–probe sets for the detection of EF. DNA bases that are relevant to the probe and reverse primer have been excluded. Horizontal lines represent DNA strands, vertical lines represent matching DNA bases. Under the figure, you will find a list of molecular markers. **c** Screening of forward primers. Image shows RPA-LFS results of different forward primers. On each lane, primer–probe pairs are indicated. Three independently conducted experiments are represented in the image. *NTC* no template control

R1B, F5/P/R1B, F6/P/R1B, and F7/P/R1B showed false-positive signal from the no template control (NTC); only F3/P/R1B was used for further detection (Fig. 1c).

Specificity of the RPA-LFS Method for Detection

To confirm the RPA-LFS method could effectively detect different *E. cloacae*, 4 reference strains and 16 sputum isolated strains were detected with the primer–probe set F3/P/R1B; this primer–probe effectively detected the pathogen (Fig. 2). To evaluate specificity of the method, 28 common pathogens (including *E. cloacae*) were detected using RPA-LFS. Only the *E. cloacae* sample was positive; the other strains were negative (Fig. 3). Thus, the primer–probe set of F3/P/R1B was used for future reactions.

Detection Limit of RPA-LFS

Using RPA-LFS, we evaluated its limit of detection. First, *E. cloacae* at 10^6–10^0 CFU per microliter was tested (1 µL for each reaction). A red weak band appeared at the test

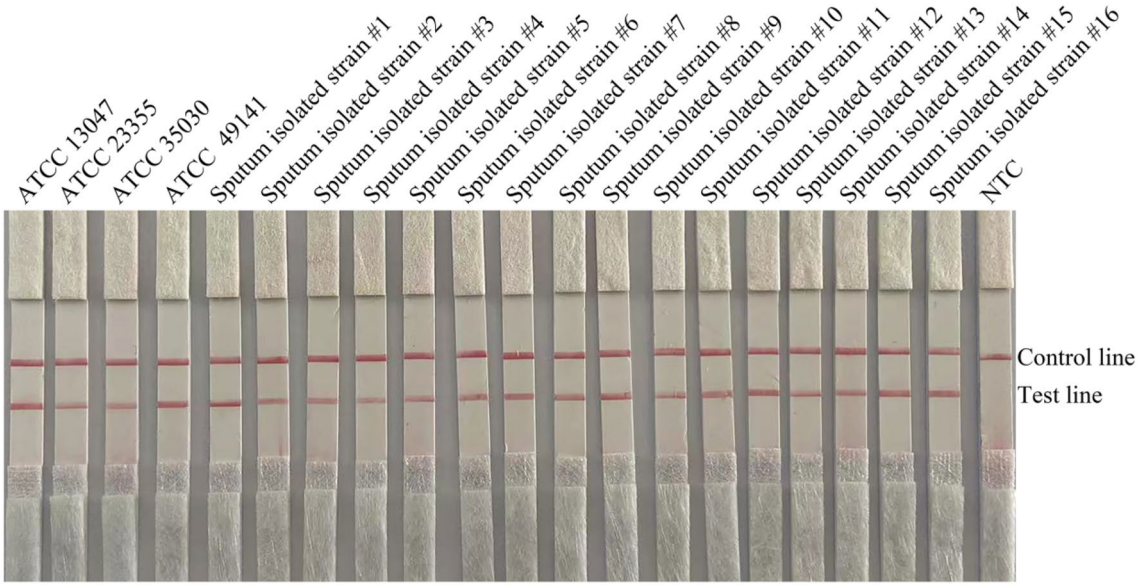

Fig. 2 Compatibility confirmation. Image shows RPA-LFS results of different reference and isolated strains of *E. cloacae*. In each lane, bacterial templates are identified by their names. Three independently conducted experiments are represented in the image. *NTC* no template control

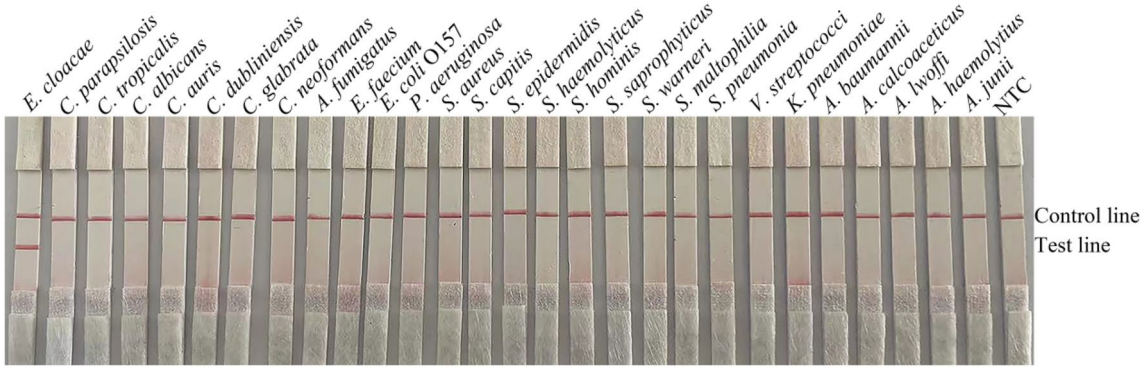

Fig. 3 Specificity confirmation. Image shows RPA-LFS results of different bacterial templates. In each lane, bacterial templates are identified by their names. Three independently conducted experiments are represented in the image. *NTC* no template control

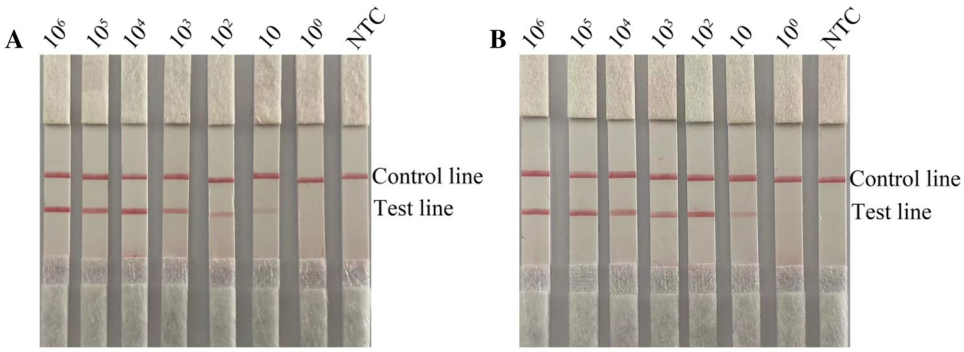

Fig. 4 Limit of detection of RPA-LFS. **a** Image shows detection results with different amounts (10^6 to 10^0 CFU/μL) of *E. cloacae*. **b** Image shows detection results with different amounts (10^6 to 10^0 CFU/μL) of *E. cloacae,* and 10^6 CFU/μL of *E. coli* O157. Reactions were performed at 37 °C for 30 min. Three independently conducted experiments are represented in the image. *NTC* no template control

Table 3 Prevalence of *E. cloacae* in 217 clinical isolates with the RPA-LFS and qPCR assays (summarized)

		RPA-LFS assay		Total
		Positive	Negative	
qPCR	Positive	73	0	73
	Negative	0	144	144
Total		73	144	217

line of 10^1 CFU. A higher CFU resulted in a stronger signal on the test line (Fig. 4)a. Second, *E. cloacae* was tenfold diluted from 10^6–10^0 CFU/μL spiked with 10^6 CFU/μL of *E. coli* O157. A signal at 10^1 CFU was observed (Fig. 4b). Thus, detection limit of RPA-LFS was 10^1 CFU/reaction.

Application of the RPA-LFS Method for *E. cloacae* Detection

A total of 217 clinical samples collected from different patients were tested for *E. cloacae* with RPA-LFS and qPCR. The detection results of RPA-LFS were consistent with those of qPCR (Table 3). Thus, RPA-LFS is a reasonable alternative for diagnosing *E. cloacae* on-site with qPCR.

Discussion

Enterobacter cloacae has emerged as a serious threat to hospitals, farms, and aquaculture worldwide. *E. cloacae* infections can cause septicemia, meningitis, endocarditis, septic arthritis, and bone marrow [4], [6]. In addition, *E. cloacae* can also cause disease in aquatic animals, resulting

in a large number of deaths [7]. These situations also occur in resource-poor regions and countries. Current methods (e.g., PCR, qPCR) cannot meet the demand in remote areas. Although LAMP is an isothermal amplification technology, it requires a temperature control instrument. Fortunately, compared to existing detection methods, RPA-LFS can compensate for their shortcomings and can be tolerated for crude templates. Human body heat can even be used to complete the reaction, so no special equipment is required [19], it is useful for the detection of *E. cloacae* in hospitals, farms, and aquaculture.

A good amplification target for effective detection of a particular species is very important; the outer membrane protein X (*omp*X) gene has been used as an amplification target in other methods [23]. Our data also indicated that the *omp*X gene had a good specificity. In addition, the primer–probe set F3/P/R1B selected in this study could effectively distinguish *E. cloacae* from other common pathogens. In the RPA-LFS method, *E. cloacae* was detected with excellent accuracy. The limitation of detection was 10^1 CFU, which is consistent with the qPCR method. This method could specifically detect *E. cloacae* in the presence of *E. coli* O157; the sensitivity was not affected in the presence of *E. coli* O157, infected patients can be identified by this method in order to determine which microorganisms are pathogenic. Importantly, the RPA-LFS method can be performed within 35 min (30 min of amplification and 5 min of LFS analysis), while the PCR and qPCR methods require more than 50 min to complete. Some areas without adequate equipment may spend more time transporting clinical samples to specialized laboratories for detection, this is not conducive to clinical treatment.

It was found that the RPA-LFS assay was just as accurate as qPCR when used on clinical samples, but did not require extraction of DNA, and it was only necessary to use bacterial liquid after high temperature lysis as a template, which means this method can be easily performed by untrained personnel.

In conclusion, its simplicity, rapidity, and accuracy made it easy to perform without the need for laboratory facilities. In combination with a simple, fast method for extracting DNA, the method may be useful for detection of *E. cloacae* on-site as well as in hospitals, farms, and aquaculture, saving both time and resources.

Conclusion

A reliable RPA-LFS method for *E. cloacae* was established after screening several primers-probe pairs. Reactions could be completed within 30 min at 37 °C, and the results could be observed on a LFS in 5 min. This method had the same limit of detection (10^1 CFU/reaction) as the qPCR method when the results of 217 clinical samples were compared. Thus, the method established in this study may be useful in remote areas or farms with poor resources.

Statement

The study protocol was approved by the Medical Ethics Committee of the Second People's Hospital of Lianyungang City (Lianyungang, Jiangsu, China) and informed consent was obtained from patients prior to collection of clinical samples. All methods were carried out in accordance with relevant guidelines and regulations.

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Author Contributions WJZ, YW, and CC designed the experiments and wrote the manuscript. JH and YW collected the clinical samples. JH, JX, and YZL performed the experiments. LW analyzed the data. All authors reviewed and approved the final version of the manuscript.

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Data Availability The data presented in this study are included in the article. Further inquiries should be directed to the corresponding authors.

Declarations

Conflict of Interest The authors have no conflicts of interest to declare.

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