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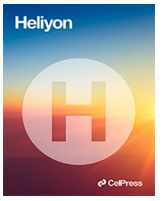

Rapid detection of *Cryptosporidium* spp. in diarrheic cattle feces by isothermal recombinase polymerase amplification assays

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ABSTRACT

As a zoonotic parasite, *Cryptosporidium* spp. could cause severe diarrhea mainly in calves and children globally. Monitoring and prevention of *Cryptosporidium* spp.'s prevalence is of great significance in both economy and public health aspects. In this study, specific primers and probes were designed within the conserved region of 18S rRNA gene for *Cryptosporidium* spp. and recombinase polymerase amplification assays based on the fluorescence monitoring (real-time RPA) as well as combined with a lateral flow strip (LFS RPA) were developed. Both of the two RPA assays allowed the exponential amplification of the target fragment within 20 min. After incubation on a metal bath at 42 °C, the LFS RPA results were displayed on the lateral flow strip within 5 min while real-time RPA allowed the real-time observation of the results in Genie III at 39 °C. The RPA assays showed high specificity for *Cryptosporidium* spp. without any cross-reaction with other tested pathogens causing diarrhea in cattle. With the recombinant plasmid DNA containing the 18S rRNA gene of *Cryptosporidium* spp. serving as a template, the limit of detection for real-time RPA and LFS RPA assays were 14.6 and 12.7 copies/reaction, respectively. Moreover, the RPA assays were validated by testing diarrheic cattle fecal samples and compared with a real-time PCR. The positive ratio of *Cryptosporidium* spp. was 24.04 % (44/183) and 26.23 % (48/183) in both RPA assays and real-time PCR assay, respectively, and the kappa coefficient value was 0.942. The diagnostic specificity and diagnostic sensitivity of both RPA assays were 100 % and 91.67 %, respectively. Forty-one of 48 positive samples were successfully sequenced and four *Cryptosporidium* species were detected, including *C. parvum* (n = 20), *C. andersoni* (n = 17), *C. bovis* (n = 3) and *C. ryanae* (n = 1). The developed RPA assays are easy to operate and faster to obtain the detection results, and they are suiting for the point-of-care detection and facilitating the prevention and control of *Cryptosporidium* spp. infections.

1. Introduction

Cryptosporidiosis is an important zoonosis and caused by *Cryptosporidium* spp., which is zoonotic parasite that belongs to the phylum Apicomplexa. *Cryptosporidium* spp. is worldwide prevalent [1–4] and exhibits genetically diverse, with over 40 species and

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more than 120 genotypes have been identified to date [5]. Cattle is one of the main reservoir hosts of *Cryptosporidium* spp [6]. Infection with *Cryptosporidium* spp. in cattle can cause severe diarrhea, affect production performance and even lead to death, resulting in huge economic losses. There are 4 species of *Cryptosporidium* that mainly infect cattle: *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* [4]. *Cryptosporidium* spp. infection is influenced by risk factors such as age, hygiene and water source [7]. *C. parvum* mainly infects pre-weaned calves and causes severe diarrhea [8,9], while *C. andersoni* frequently infects adult cattle and was considered to be associated with gastritis as well as milk yield reduction [10]. *C. bovis* and *C. ryanae*, by contrast, mainly infect post-weaned cattle and may induce the occurrence of diarrhea [9,11]. Presently, a variety of non-dominant *Cryptosporidium* species had been identified in cattle, e.g. *C. ubiquitum*, *C. meleagridis*, *C. hominis*, *C. xiaoi*, *C. suis*, *C. serpentis*, *C. muris*, *C. felis* and *C. scrofarum* [12]. Moreover, a large number of *Cryptosporidium* species infecting animals have potential to be zoonotic [13]. *Cryptosporidium* spp. had triggered several outbreaks that pose a serious threat to human health [14–16]. The effective prevention and control of *Cryptosporidium* spp. has become urgent. However, there is no effective vaccine against *Cryptosporidium* spp. while many drugs have been tested and found to be less than effective [17]. Therefore, rapid, accurate and sensitive detection methods will be essential to achieve epidemiological surveillance of *Cryptosporidium* spp. and to provide strong support for disease prevention and control.

In recent years, a variety of diagnostic tools for *Cryptosporidium* spp. have been reported. Traditional microscope-based detection methods have long been regarded as the gold standard, but they are time-consuming, labor-intensive and poorly sensitive, requiring professional operation [18]. Antigen-based detection methods, including direct fluorescent antibody test (DFAT) and enzyme-linked immunosorbent assay (ELISA), are simple as well as inexpensive and have been widely used in the detection of considerable clinical samples, but they are prone to false positives and most ELISA commercial kits can only target single *Cryptosporidium* specie rather than all *Cryptosporidium* species [19,20]. PCR-restriction fragment length polymorphism (RFLP) allows specific detection of various *Cryptosporidium* species [21]. Compared to RFLP, real-time PCR increases the sensitivity and dramatically reducing detection time [18, 22]. However, the PCR-based detection methods rely on expensive thermal cycling instruments and specialized laboratory environments, which limits the application in the field or resource-limited settings. Loop-mediated isothermal amplification (LAMP) is an isothermal amplification technique that discard complex thermal cycling instruments and amplify the target fragment at a constant temperature. However, it requires four primers to achieve amplification at 63 °C for 60 min [23]. These factors, coupled with frequent false positives, limit its use in clinical testing.

Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification technique that has been widely used to detect different kinds of pathogens [24–26]. RPA can achieve exponential amplification of the target fragment in less than 30 min at a lower temperature range (25–42 °C) [27,28]. RPA has three mainly assay forms, i.e. amplification by specific primers and endpoint detection after purification, display on the portable fluorescence scanner device or lateral flow strip (LFS) by adding simple modified exo or nfo probe to the reaction system [27,29]. Compared with the above diagnostic methods, RPA has the advantages of rapid amplification, convenient operation, simple instrument requirement and diverse display forms, showing huge potential for application in point-of-care detection.

The 18S rRNA gene is a multi-copy gene that facilitates the sensitivity of the assay and has been the target gene of a number of molecular diagnostic tools for *Cryptosporidium* spp [3]. In this study, specific RPA primers, exo probe and nfo probe based on the conserved sequence of 18S rRNA gene were designed, and the real-time RPA and LFS RPA assays were developed for the rapid, simple, and reliable detection of *Cryptosporidium* spp. in diarrheic cattle fecal samples.

2. Materials and methods

2.1. Pathogen strains and clinical samples

The oocysts of *C. parvum* (C. parvum/HB/HD), *C. andersoni* (C. andersoni/HB/BD), *C. bovis* (C. bovis/HB/HS) and the DNA of

Table 1
Detection of *Cryptosporidium* spp. in fecal samples using the real-time RPA, LFS RPA, real-time PCR and nested PCR.

Locations	Monthly age	Number of samples	Results			
			real-time RPA	LFS RPA	real-time PCR	nested PCR
Shijiazhuang	< 3	7	2	2	2	2
	> 18	29	4	4	4	4
Baoding	< 3	19	6	6	8	6
	> 18	22	1	1	1	1
Zhangjiakou	< 3	22	11	11	11	8
	> 18	12	0	0	0	0
Hengshui	< 3	7	3	3	3	3
	> 18	11	5	5	5	5
Handan	< 3	10	4	4	4	4
Chengde	> 18	10	5	5	5	5
Cangzhou	> 18	7	3	3	5	3
Langfang	< 3	23	0	0	0	0
Xingtai	< 3	2	0	0	0	0
	> 18	2	0	0	0	0
Total		183	44	44	48	41

Eimeria zuernii (*E. zuernii*, HB/BD-1), *Eimeria bovis* (*E. bovis,* HB/HS-1), *Escherichia coli* K99^{+} (*E. coli* K99^{+}, HB/HS6), *Salmonella Typhimurium* (CICC 22956), *Clostridium perfringens* (CICC 22949) and *Mycobacterium avium. ssp. paratuberculosis* (MAP, HB/HD2) as well as the cDNA of Bovine rotavirus A (BRVA, HB-LF/2021) were reserved in our laboratory. One hundred and eighty-three fecal samples from the cattle with severe diarrhea were collected from 28 different cattle farms in 9 different regions of Hebei province between May 2021 and August 2022. All cattle were divided into two groups according to age: calves under three months (n = 90) and adult cattle over 18 months (n = 93) (Table 1). The fecal samples were obtained by dipping intra-anal using a sterile swab or collecting with sterile gloves immediately after defecation. All samples were stored in sterile 50 ml non-pyrogenic DNase/RNase Free centrifuge tubes and transported at 4 °C.

2.2. DNA extraction

The oocysts of *Cryptosporidium* stored in 2.5 % potassium dichromate were washed five times with distilled water to remove the preservative by shaking and centrifugation before DNA extraction. Then 200 μL oocysts suspension and 200 mg of normal fecal or 200 μL of watery fecal were added to a 2.0 ml centrifuge tube, and the genome DNA was extracted using the TIANamp Stool DNA kit (Tiangen, Beijing, China) following the manufacturer's instructions. The extracted DNA were quantified using ND-2000c spectrophotometer (NanoDrop, Wilmington, USA). All DNA templates were used immediately or stored at −80 °C until use.

2.3. Construct of standard plasmid

The genome DNA of *C. parvum* was used as a template to amplify a fragment of 834 bp of the 18S rRNA gene by using the PCR primers (nPCR-F2/nPCR-R2, Table 2) from a previously described protocol [21]. The PCR products were purified using the TIANgel Midi Purification Kit (Tiangen, Beijing, China) and ligated into a pTOPO-TA vector (Aidlab, Beijing, China). The constructed plasmid transformed into *E. coli* DH5α chemically competent cells for cloning. The plasmid DNA extracted from *E. coli* was verified by sequencing, quantified using ND-2000c spectrophotometer (NanoDrop, Wilmington, USA) and calculated the copy number of DNA molecules by the previously published formula [24]. Serving the following analysis, the plasmid DNA was serially diluted 10-fold with RNase-free ddH_2O, ranging from 1.6×10^5 to 1.6×10^{-1} copies/μL.

2.4. Primers and probes of the RPA assays

The highly conserved 18S rRNA gene among the different *Cryptosporidium* species was set as the target of the RPA assays. The partial or complete sequences of 30 different *Cryptosporidium* species were obtained from the GenBank and aligned using the DNASTAR (DNASTAR, Madison, USA) to identify the conserved region. The primers and probes were designed based on the most conserved region referring to *C. parvum* (AF093490) (Fig. S1). Two forward primers and two reverse primers were first designed and combined into four pairs. The most suitable pair of the four pairs was used as candidate primers for optimization. The exo probe and nfo probe

Table 2
Sequences of the primers and probes for *Cryptosporidium* spp. nested PCR, real-time PCR, real-time RPA and LFS RPA assays.

Assays	Primers and probes	Sequence (5′~3′)	Location	Reference
nested PCR	nPCR-F1	TTCTAGAGCTAATACATGCG	155~174	(Xiao et al., 2000)
	nPCR-R1	CCCATTTCCTTCGAAACAGGA	1453~1473	
	nPCR-F2	GGAAGGGTTGTATTTATTAGAT AAAG	192~217	
	nPCR-R2	AAGGAGTAAGGAACAACCTCCA	1004~1025	
real-time PCR	CcF18S	GTTTTCATTAATCAAGAACGAAAGTTAGG	916~944	(Burnet et al., 2013)
	CcR18S	GAGTAAGGAACAACCTCCAATCTCTAG	996~1022	
	Csp18S	FAM-TCAGATACCGTCGTAGTCTTAA CCATAAACTATGCC-BHQ1	958~993	
RPA	18S1F	TTCAAGTTTCTGACCTATCAGCTTTAGACGGT	277~308	This study
	18S1R	CTTGTAAAGGGGTTTATACTTAACTCATTCC	488~518	
	18S2F	GACCTATCAGCTTTAGACGGTAGGGTATTG	288~317	
	18S2R	CTGTATTGTTATTTCTTGTCACTACCTCCCTG	436~467	
	18S1F-1	**CATTCAAGTTTCTGACCTATCAGCTTTAGAC**	**275~305**	
	18S1F-2	ATCATTCAAGTTTCTGACCTATCAGCTTTA	273~302	
	18S1F-3	CATATCATTCAAGTTTCTGACCTATCAGCT	270~299	
	18S1R-1	GATACTTGTAAAGGGGTTTATACTTAACTC	493~522	
	18S1R-2	**CTTGTAAAGGGGTTTATACTTAACTCATTC**	**489~518**	
	18S1R-3	ATTGATACTTGTAAAGGGGTTTATACTTAACTC	493~525	
	18s-exoP	**GCAATGACGGGTAACGGGGAATTAGGGT(FAM-dT)C(THF)A(BHQ1-dT) TCCGGAGAGGGAGC-C3-spacer**	**328~374**	
	LFS18S1R-2	**Biotin-CTTGTAAAGGGGTTTATACTTAACTCAT TC**	**489~518**	
	18s-nfoP	**FAM-CAATCCTAATACAGGGAGGTAGTGAC AAGA(THF)ATAACAATACAGGAC-C3-spacer**	**425~470**	

Note: The location of primers/probes refers to the position in the 18S rRNA gene of a *C. parvum* strain Bovine C. parvum genotype (BOH6) (GenBank accession no. AF093490).

were designed based on the optimal primer pair screened. Besides the primers and probes designed in this study, the nested PCR primers, real-time PCR primers and TaqMan probe from the previously described protocols [21,22] were also synthesized by a commercial company (Generay, Shanghai, China) and shown in Table 2.

2.5. Real-time RPA assay

The real-time RPA assays for *Cryptosporidium* spp. were performed using a ZC BioScience™ exo kit (ZC BioScience, Hangzhou, China). Each single reaction system contained 25 μL of Buffer A (rehydration buffer), 2.0 μL of each RPA primer (10 μmol/L), 0.6 μL of exo probe (10 μmol/L), 2.5 μL of Buffer B (magnesium acetate). In addition, depending on the different analyses, 1 μL of control plasmid DNA or 2 μL of sample DNA was added and the total volume was made up to 50 μL with the RNase-Free ddH_2O. According to the manufacturer's instructions, the Buffer A, primers, exo probe and the appropriate amount of ddH_2O were configured into a mixture depending on the amount of reaction required, and dispensed into a 0.2 ml freeze-dried reaction tube containing a dried enzyme pellet, then the template was added and the tube lid with 2.5 μL of Buffer B on the inside was capped. The magnesium acetate was centrifuged into the solution by minispin centrifuge, mixed thoroughly by flicking the wall of the tube or vortex shaking, and then the tubes were immediately placed in the Genie III scanner device (OptiGene Limited, West Sussex, UK) to start the reaction at 39 °C for 20 min.

2.6. LFS RPA assay

The LFS RPA assays for *Cryptosporidium* spp. were performed using a GenDx ERA Kit (GenDx Biotech, Suzhou, China). The total volume of each reaction was 50 μL, which included 20 μL of Buffer A, 2.1 μL of each RPA primer (10 μmol/L), 0.6 μL of nfo probe (10 μmol/L), and 2.0 μL of Buffer B. Like the operation of real-time RPA, all the ingredients were mixed together except for the template DNA and Buffer B, and dispensed into a 0.2 ml freeze-dried reaction tube, then the template was added and the tube lid with 2.0 μL of Buffer B on the inside was capped. After thorough mixing as real-time RPA assays, the reaction tubes were incubated in a thermostatic metal bath. The 5 μL LFS RPA product was diluted with 200 μL of ddH_2O. The lateral flow strips (GenDx Biotech, Suzhou, China) were placed in the diluent and the results were analyzed within 5 min.

2.7. Optimization of incubation temperature and time of LFS RPA

To screen the optimal reaction temperature for the LFS RPA assay, the reaction tubes were incubated for 30 min at 35–45 °C with an interval of 1 °C using Gradient PCR instruments (Applied Biosystems, Foster City, California), respectively. The analysis of each temperature was performed in parallel. To screen the optimal reaction time, five reactions were incubated separately for different duration (10, 15, 20, 25, 30 min) at the optimal temperature. Both optimization analyses were performed using the standard plasmid DNA of 1.6×10^2 copies/μL as the template and repeated three times.

2.8. Analytical specificity and sensitivity analysis

The screened primers and probes were used to amplify a range of pathogens which cause diarrhea in cattle to verify the specificity of the developed RPA assays for *Cryptosporidium* spp. Briefly, 10 ng DNA of *C. parvum*, *C. andersoni*, *C. bovis*, *E. zuernii*, *E. bovis*, *E. coli* $K99^+$, *Salmonella Typhimurium*, *Clostridium perfringens*, MAP and the cDNA of BRVA were used as the template to amplify in the both RPA assays. Meanwhile, the nucleic acid extracted from *Cryptosporidium*-negative feces was used as negative control and ddH_2O was used as non-template control. All of the analytical specificity analyses were repeated five times. The analytical sensitivity of the RPA assays was confirmed by amplifying $1.6 \times 10^5 \sim 1.6 \times 10^{-1}$ copies/μL of the standard plasmid DNA. In addition, 2-fold dilution series with 1.6×10^1 copies/μL of template was performed until 1.6×10^0 copies/μL and also used as template for analytic sensitivity. The ddH_2O was added as non-template control to verify the absence of cross contamination. All analytical sensitivity analyses were repeated eight times on different days by different operators. Based on the detection frequency of each concentration template in eight replications, the Probit analyses were performed to determine the limit of detection (LOD) of the developed RPA assays by using SPSS software (v22.0, Armonk, USA).

2.9. Real-time PCR assay

The DNA extracted from 183 bovine fecal samples was used as the template to analyzed *Cryptosporidium* spp. positive ratio by using the real-time PCR primers (CcF18S/CcR18S) and TaqMan probe (Csp18S) from a previously described protocol [22]. The TAMRA quencher of the TaqMan probe was changed to BHQ1 in this study and real-time PCR assay was operated using conditions optimized by our laboratory. Briefly, each reaction was consisted of 12.5 μL of 2 × Go Taq® Probe qPCR Master Mix (Promega, Madison, USA), 2 μL of sample DNA, 1.0 μL of each primer and probe (10 μmol/L) and 7.5 μL of RNase-Free ddH_2O. The reaction condition was set as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All real-time PCR assays were performed on ABI Quant Studio 5 (Applied Biosystems, Foster City, California).

2.10. Validation with clinical samples

The developed real-time RPA and LFS RPA assays were used to detect 183 fecal samples from cattle with diarrhea. The 2 μL of

genome DNA extracted from the sample was used as the template for amplification. The results of the RPA assays were compared with real-time PCR to evaluate their applicability in clinical diagnosis.

2.11. Sequence and phylogenetic analysis

The nested PCR was performed on all positive samples according to a previously published protocol using primers (nPCR-F1/nPCR-R1 and nPCR-F2/nPCR-R2) [21]. The PCR products showing the target fragment were sequenced directly in both directions by a commercial company (Tsingke Biotechnology Co., Ltd., Beijing, China) using the secondary PCR primers. All sequences obtained were confirmed as *Cryptosporidium*-related sequences by BLAST (https://blast.ncbi.nlm.nih.gov/) and homologies were analyzed of all sample sequences using the MegAlign software (version 7.0; DNASTAR Inc., WI, USA). Retrieved 18S rRNA gene sequences of *Cryptosporidium* species from GenBank and use them as reference sequences. Both the positive sample sequences and reference sequences were performed a multiple sequence alignment by ClustalW as well as a phylogenetic tree with 1000 bootstraps support was constructed with the sequences of *E. bovis* (KT184336) and *Colpodella* sp. (AY449717) as outgroups by using the neighbor-joining method in the MEGA 6.05 software.

3. Results

3.1. Screening of the optimal primer-probe combinations

Firstly, two forward primers (18S1F, 18S2F) and two reverse primers (18S1R, 18S2R) were designed and combined into four combinations. The primary primer was screened by the basic RPA assay and the purpose band of the four pairs of primers did not differ on 2.0 % agarose gel (Fig. S2A). 18S1F/18S1R were chosen for the second round screening, which amplifies the longer fragments for subsequent screening due to the large number of duplicates and palindromic sequences in the 18S rRNA gene. Surrounding the 18S1F/18S1R, three additional forward primers (18S1F-1, 18S1F-2, 18S1F-3) and reverse primers (18S1R-1, 18S1R-2, 18S1R-3) were designed by move back and forth or add and delete sequences. Together with 18S1F/18S1R, the four forward primers and four reverse primers were combined into 16 combinations, and again screened by the basic RPA using the standard plasmid DNA with 1.6×10^3

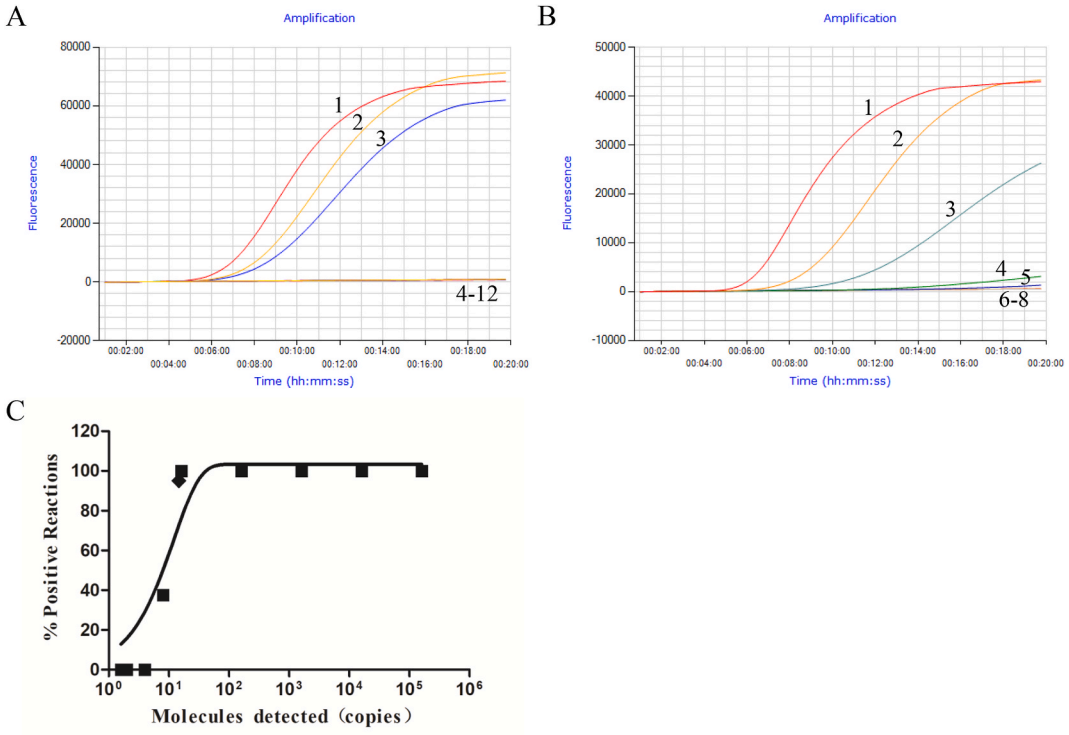

Fig. 1. Performance of *Cryptosporidium* spp. real-time RPA assay. (A) Evaluation of the analytical specificity of the real-time RPA assay. Line 1, *C. parvum*; line 2, *C. andersoni*; line 3, *C. bovis*; line 4, *E. zuernii*; line 5, *E. bovis*; line 6, *E. coli* K99$^+$; line 7, *Salmonella Typhimurium*; line 8, *Clostridium perfringens*; line 9, MAP; line 10, BRVA; line 11, negative control; line 12, ddH_2O. (B) Evaluation of the analytical sensitivity of the real-time RPA assay. Line 1, 1.6×10^5copies; line 2, 1.6×10^4copies; line 3, 1.6×10^3copies; line 4, 1.6×10^2copies; line 5, 1.6×10^1copies; line 6, 1.6×10^0copies; line 7, 1.6×10^{-1}copies; line 8, ddH_2O. (C)Probit regression analysis of the real-time RPA assay using the data of the detection rate from each concentration of the eight replicates. The limit of detection at 95 % probability (14.6 copies/reaction) is depicted by a rhomboid.

copies/μL as the template (Fig. S2B). 18S1F-1/18S1R-2 that produced the brightest bands were chosen and the exo probe was synthesized based on 18S1F-1/18S1R-2. The addition of probes may affect amplification efficiency, so primers with similar amplification efficiency in the basic RPA assays were screened again by real-time RPA assays. 18S1F-1/18S1R-2 and the 18s-exoP combination produced the lowest threshold time (TT) value and the highest fluorescence value were used in the following real-time RPA assay (Fig. S2C). In general, the sequence of exo probe could be used directly on nfo probe, whereas the nfo probe using the same sequence as the exo probe were observed to produce non-specific binding with reverse primer in the LFS RPA assay, thus, another nfo probe (18s-nfoP) was designed with different sequences.

3.2. Performance of the real-time RPA assay

For the real-time RPA assays, the presence of a typical fluorescence curve within 20 min is considered positive. In the analytical specificity analysis, the amplification curves were only observed in the lanes of *C. parvum*, *C. andersoni* and *C. bovis* while the amplification curves were not observed in the other pathogens, negative control and ddH_2O (Fig. 1A). Five identical results revealed the high specificity of the real-time RPA assay for *Cryptosporidium* spp. In the analytical sensitivity analysis, the amplification curves were always observed for the standard plasmid of 1.6×10^5~1.6×10^1 copies/μL while the amplification curves were never observed for 1.6×10^0, 1.6×10^{-1} copies/μL and ddH_2O in all eight runs (Fig. 1B). Using the 0.8×10^1copies/μL concentration of template, the curves were observed in three of the eight replicates; using the 0.4×10^1, 0.2×10^1, 1.6×10^0 copies/μL concentration of templates, the curves were never observed in all eight replicates. The probit regression analysis revealed that the LOD of the real-time RPA assay was 14.6 copies/reaction (95 % CI: 11.5 to 28.6 copies/reaction) (Fig. 1C).

3.3. Optimization of the LFS RPA assay

For the LFS RPA assays, the result was judged as positive when the control line and the test line were striped in the lateral flow strips, and the result was judged as negative when the control line was observed and the test line did not. In the temperature optimization analysis, the test line was observed from 10 temperatures of 35~45 °C while the test line was clearest at 42 °C (Fig. 2A). In the time optimization analysis, a very weak band was observed after 10 min of incubation. As the reaction time increased the test line became more visual, whereas the test line did not display distinct difference within 20–30 min (Fig. 2B). All three repetitions showed the same result and the optimal incubation conditions of the LFS RPA assay was confirmed to be at 42 °C for 20 min.

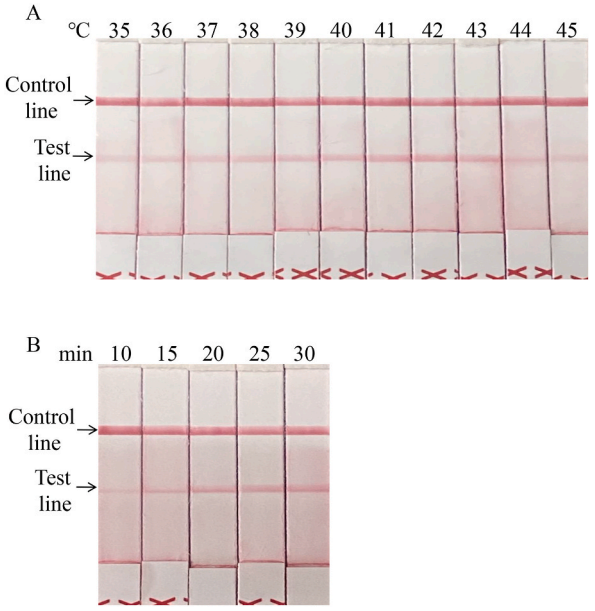

Fig. 2. Optimization of the *Cryptosporidium* spp. LFS RPA assay. (A) Optimization of LFS RPA reaction temperature. The target gene of *Cryptosporidium* spp. was amplified successfully at 35 °C–45 °C. The red bands (test lines) were visible at 35 °C–45 °C, and the test line band was clearest at 42 °C, which was set as the optimal reaction temperature. (B) Optimization of LFS RPA incubation time. The target gene of *Cryptosporidium* spp. was amplified successfully at 42 °C for 10 miñ30 min. The red bands (test lines) were clearer after incubation for 20 min and 30 min, and there were no clear differences for them. The optimal reaction time was set as 20 min. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Performance of the LFS RPA assay

In the analytical specificity analysis, the test lines were always observed for *C. parvum*, *C. andersoni* and *C. bovis* while the test lines were never observed for the other pathogens, negative control and ddH_2O (Fig. 3A). Five replicates showed the same high specificity as real-time RPA assay. In the analytical sensitivity analysis, the test lines were always observed for the standard plasmid of 1.6×10^5~1.6×10^1 copies, while the test line was not observed neither for 1.6×10^0 and 1.6×10^{-1} copies nor ddH_2O in all eight runs (Fig. 3B). The test lines were observed in five and three times of the eight replicates using templates at concentrations of 0.8×10^1 and 0.4×10^1copies/μL, respectively; using the 0.2×10^1 and 1.6×10^0 copies/μL concentration of templates, the test lines were never observed in all eight replicates. According to Probit regression analysis, the LOD for the LFS RPA assay was 12.7 copies/reaction (95 % CI: 9.5 to 25.0 copies/reaction) (Fig. 3C).

3.5. Validation of the RPA assays on clinical samples

The positive ratio of *Cryptosporidium* spp. was 24.04 % (44/183) in both RPA assays while positive ratio was 26.23 % (48/183) in real-time PCR assay. Of the 48 positive samples in the real-time PCR, 44 were positive and the other 4 were negative in the both RPA assays (Table 1). Compared with real-time PCR assay, the diagnostic specificity and sensitivity of the both RPA assays was 100 % and 91.67 %. The overall diagnostic agreement between the RPA assays and real-time PCR was 97.81 % (179/183) and the kappa coefficients were 0.942 (Table 3). The TT of real-time RPA and cycle threshold (Ct) of real-time PCR values showed a linear relationship with the R^2 value of 0.7937 (Fig. S3). In the both RPA assays, the positive ratio was 28.89 % (26/90) in calves and 19.35 % (18/93) in adult cattle.

3.6. Sequence and phylogenetic analysis

Forty-one of 48 positive samples were successful amplified by nested PCR and sequenced (Table 1). The sequences of same *Cryptosporidium* species revealed high 100 % identity in each region. Eleven partial 18S rRNA sequences of *Cryptosporidium* spp. represent strains from different regions had been submitted to GenBank. Both 11 represent strains sequences and reference sequences were performed a multiple sequence alignment and a phylogenetic tree was constructed. The phylogenetic tree showed that samples from the Hengshui included *C. andersoni*, *C. bovis*, and *C. ryanae*, samples from Shijiazhuang and Baoding included *C. parvum* and *C. andersoni*, samples from Chengde and Cangzhou included only *C. andersoni*, samples from Zhangjiakou and Handan included only *C. parvum* (Fig. 4). Further analysis revealed 41 *Cryptosporidium* spp. sequences including *C. parvum* (n = 20), *C. andersoni* (n = 17), *C. bovis* (n = 3) and *C. ryanae* (n = 1). The GenBank accession numbers are ON982289, ON982450, ON982451, ON986849, ON986850, ON986851, ON986971, OP132538, OP133409, OP179659, OP210755.

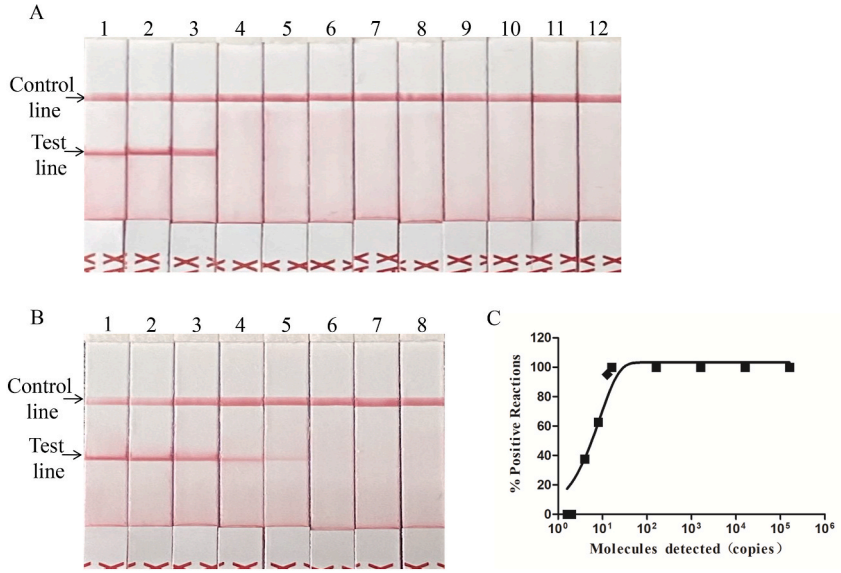

Fig. 3. Performance of *Cryptosporidium* spp. LFS RPA assay. (A) Evaluation of the analytical specificity of the LFS RPA assay. Line 1, *C. parvum*; line 2, *C. andersoni*; line 3, *C. bovis*; line 4, *E. zuernii*; line 5, *E. bovis*; line 6, *E. coli* $K99^+$; line 7, *Salmonella Typhimurium*; line 8, *Clostridium perfringens*; line 9, MAP; line 10, BRVA; line 11, negative control; line 12, ddH_2O. (B) Evaluation of the analytical sensitivity of the LFS RPA assay. Line 1, 1.6×10^5copies; line 2, 1.6×10^4copies; line 3, 1.6×10^3copies; line 4, 1.6×10^2copies; line 5, 1.6×10^1copies; line 6, 1.6×10^0copies; line 7, 1.6×10^{-1}copies; line 8, ddH_2O. (C)Probit regression analysis of the LFS RPA assay using the data of the detection rate from each concentration of the eight replicates. The limit of detection at 95 % probability (12.7 copies/reaction) is depicted by a rhomboid.

Table 3
Diagnostic sensitivity, diagnostic specificity, predictive value, and kappa value of the RPA assays for *Cryptosporidium* spp. compared to the real-time PCR.

		real-time PCR		
		P	N	T
RPA assays	P	44	0	44
	N	4	135	139
	T	48	135	183
		DSe:91.67 %	DSp:100 %	K:0.942
		PPV:100 %	NPV:97.12 %	

Note: P, positive; N, negative; T, total; DSe, diagnostic sensitivity; DSp, diagnostic specificity; K, kappa coefficient value; PPV, positive predictive value; NPV, negative predictive value.

4. Discussion

Cryptosporidiosis has a significant impact on the development of cattle industry worldwide [1]. In this study, we aligned the 18S rRNA gene sequences of 30 different *Cryptosporidium* species, then designed primers and probes in conserved regions and developed the real-time RPA and LFS RPA assays for *Cryptosporidium* spp. Even though only three *Cryptosporidium* species were used in the applicability analysis of the developed methods, the primers and probes sequences have only 0–5 base mismatches with all species except for *C. struthionis*. RPA has been reported to tolerate mismatches of 6∼9 bases [30,31]. Therefore, both of the RPA assays are deemed to amplify these species theoritically. For the *C. struthionis*, the reverse primer designed in this study had mismatches of 10 bases with the unique complete sequence on the NCBI, but this is not surprising as it is distantly related to other *Cryptosporidium* species and our phylogenetic tree shows it to be in the same branch as *Colpodella* sp. Currently, cattle had been reported to be infected with *C. struthionis* in China [32], however, the performance of the RPA assays developed in this study for *C. struthionis* needs to be further studied.

The difficulty of RPA technology is that there is no dedicated software for designing primers presently, and a pair of high-performance primers was usually identified by screening extensively [25]. In this study, we performed two rounds of screening and compared the sensitivity of 16 pairs of primers to achieve the best detection performance. The LOD for real-time RPA and LFS RPA assays were 14.6 and 12.7 copies/reaction, respectively, which are similar to the previously published real-time PCR but the RPA assays are faster to obtain the results [22]. Compare to previously published RPA-based LF-RPA and RPAC assays, the developed RPA assays have similar detection performance, but provide a fluorescent signal detection forms that facilitate real-time observation of the assay results [33,34]. The 18S rRNA gene has mostly conserved regions among eukaryotes while primers and probes designed to target conserved regions will result in cross-amplified [35,36]. Recent reports have shown that *Colpodella* sp. have a high homology (97.26 %) with *Cryptosporidium* spp. and cross-amplified had been observed in animal fecal samples [35]. The primers and probes of the developed RPA assays have a large number of mismatches with multiple *Colpodella* sp. sequences (AY142075, AY449717, AF330214, KT600662, MN640805) by BLAST. Several reports had shown that a large number of base mismatches can significantly affect the amplification efficiency of RPA, we theoretically hypothesized that the developed RPA assays could not amplify the nucleic of *Colpodella* sp [37,38]. In addition, the developed RPA assays demonstrated good specificity for *Cryptosporidium* spp. and no cross-reactivity for other common bovine diarrhea pathogens.

Over the past three decades, *Cryptosporidium* spp. has spread widely across the country [4,9,12]. The developed RPA assays were used to detect 183 diarrheic cattle fecal samples collected from nine regions in Hebei province and compared with a developed real-time PCR [22]. In the both RPA assays, the positive ratio was 24.04 % (44/183). Apart from Langfang and Xingtai, the positive ratio for *Cryptosporidium* spp. ranged from 16.67 % (6/36) to 50.00 % (5/10) in the other seven regions of Hebei province. The differences in positive ratio depend on the farm's control measures and the number of samples collected. From the perspective of cattle age, 26 (28.89 %) were obtained from calves and 18 (19.35 %) were obtained from adult cattle, indicating that *Cryptosporidium* spp. is prevalent in calves and adult cattle on cattle farms. The consistent rate of the RPA assays and real-time PCR was 97.81 % (179/183). The Ct values of 4 samples were 20.37–25.60, however, in a further study, all the 4 samples showed negative results by the nested PCR. indicating the possible existence of non-specific amplification in the real-time PCR. This may be due to the fact that the feces contain a variety of fungal and parasitic, and the real-time PCR primers and probe may mistakenly identify the DNA of organisms with similar sequences to *Cryptosporidium* spp.

Surprisingly, three of the 44 RPA positive samples were negative in nested PCR, which contained large amounts of *Cryptosporidium* spp. DNA with TT values of 5:08∼6:20. Furthermore, three RPA products were sequenced after DNA purification, and the 246 bp fragment exhibited 100 % homology with *C. parvum*, which were confirmed as *Cryptosporidium*-related sequences by BLAST. According to some reports of the effect of PCR inhibitors on PCR reactions [21,39,40], we attribute the inconsistency in detection rates between nested PCR and RPA assays to the presence of inhibitors in the feces. Forty-one partial sequences of *Cryptosporidium* spp. 18S rRNA gene from all regions were obtained by sequencing and *Cryptosporidium* species were identified by phylogenetic tree analysis. Further analysis demonstrated that *C. parvum* dominated in calves while *C. andersoni* dominated in adult cattle (data not shown) in this study, which was consistent with the previous reports [8–10].

Cryptosporidium spp. is mainly transmitted through drinking water and food [41–43], so point-of-care detection assay will be conducive to epidemic prevention and control to the greatest extent. RPA is unaffected by PCR inhibitors like bilirubin, bile salts,

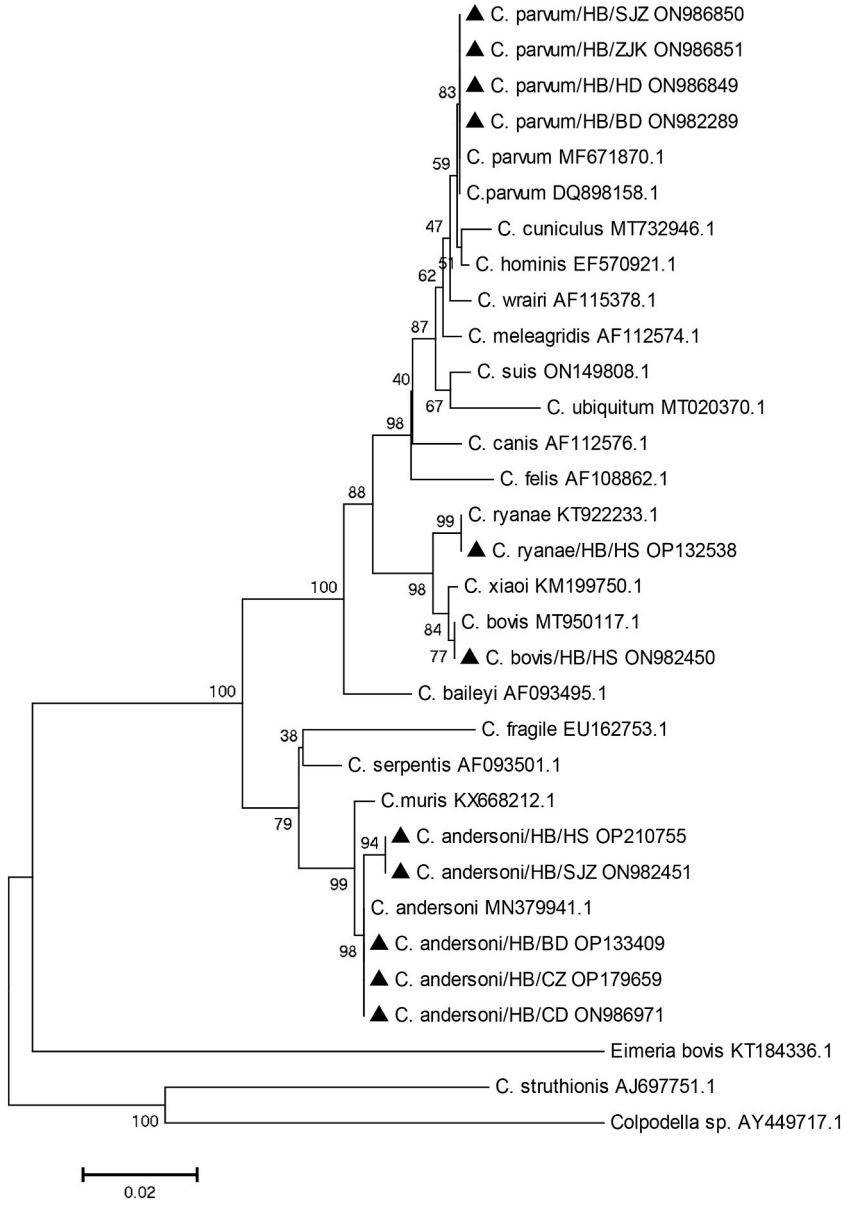

Fig. 4. Phylogenetic analysis of the *Cryptosporidium* spp. positive strains based on the nucleotide sequences of their about 830 bp partial 18S rRNA genes. The phylogenetic tree was constructed by the neighbor-joining clustering method with 1000 bootstrap replicates, using MEGA version 6.05 software. The representative strains reported in this study is denoted by black triangles. All accession numbers used in the phylogenetic analysis had been labeled in the figure.

humic acids and other contaminants and can be used to detect samples with complex composition such as sewage, fecal and offal [31]. It has been reported that fecal samples can be used to detect *Cryptosporidium* spp. with a simple incubation through the RPA assays [34], which means that RPA can really achieve point-of-care by combining with some rapid nucleic acid extraction kits. At present, our laboratory is comprehensively comparing the performance of the various commercial kits and is committed to finding a suitable kit for rapid extraction of *Cryptosporidium* spp. nucleic acid in fecal samples.

It is worth noting that not only RPA has the advantages of rapid response, simple operation, and independence of complex instruments, but also its reagents can be stored at room temperature for a long time [44], which greatly increases its scope of application. Interestingly, the LFS RPA can be incubated for amplification by hand-holding and a disposable hand warmer bag [26]. Moreover, the real-time RPA product can produce fluorescence under 480 nm wavelength light irradiation [25]. In this study, the real-time RPA product in the sensitivity analysis produced a clear gradient fluorescence signal by multicolor imaging using the gel imaging system (Fig. S4). All these extremely simple operations make the RPA assays more flexible for point-of-care detection.

In conclusion, we established the real-time RPA and LFS RPA assays for rapid detection of *Cryptosporidium* spp. in cattle feces. Both

of the RPA assays are highly specific and sensitive which have a great significance for monitoring *Cryptosporidium* spp. epidemics and controlling the spread of *Cryptosporidium* spp.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

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CRediT authorship contribution statement

Yuelin Liu: conceptualized and designed the experiments, and. **Jialin Xiang:** collected the samples and performed the experiments. **Yaxin Gao:** and. **Jinfeng Wang:** and. **Libing Liu:** collected the samples and analyzed the data. **Ruiwen Li:** wrote and revised the manuscript, All authors contributed to the article and approved the submitted version. **Jianchang Wang:** and, and.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20794.

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