International Journal of Infectious Diseases 86 (2019) 108–113

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

A rapid and sensitive recombinase aided amplification assay incorporating competitive internal control to detect *Bordetella pertussis* using the DNA obtained by boiling

Rui-qing Zhang[a,b,1], Gui-xia Li[c,1], Xin-na Li[b], Xin-xin Shen[b], Yuan Gao[d], Le Wang[c], Tao Fan[a,b], Qing-xia Duan[a,b], Ya-kun Wang[c], Ji Wang[b,*], Zhi-shan Feng[e,**], Xue-jun Ma[b,*]

[a] *Hebei Medical University, Shijiazhuang, 050031, Hebei, China*
[b] *NHC Key Laboratory of Medical Virology and Viral Diseases, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155, Changbai Street, Changping District, Beijing 102206, China*
[c] *Children's Hospital of Hebei Province, Shijiazhuang, 050031, Hebei, China*
[d] *National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, National State Key Laboratory for Infectious Disease Prevention and Control, Beijing, 102206, China*
[e] *Hebei General Hospital, Shijiazhuang, 050051, Hebei, China*

ARTICLE INFO

Article history:
Received 11 May 2019
Received in revised form 22 June 2019
Accepted 29 June 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Recombinase aided amplification
Bordetella pertussis
Internal amplification control

ABSTRACT

Objectives: Pertussis is a highly transmissible acute respiratory infection caused by the bacterial pathogen *Bordetella pertussis*. The purpose of this study was to develop a rapid, simple and sensitive diagnostic test for detecting this pathogen.

Methods: Here we present a recombinase aided amplification (RAA) assay incorporating competitive internal amplification control (IAC) to detect *Bordetella pertussis* using the DNA obtained by boiling. This assay was performed in a single closed tube at 39 °C within 30 min. A total of 115 clinical samples suspected of pertussis were collected and tested by the internally controlled RAA assay using both extracted DNA with the commercial kit and the DNA obtained by boiling. For comparison, the real-time PCR (RT-PCR) was also performed with DNA extraction in parallel.

Results: The sensitivity of the internally controlled RAA assay was 10^1 copies or 10 CFU/ml per reaction in detecting plasmid DNA or *B. pertussis* strain. The optimum concentration of the IAC plasmid was determined to be 100 copies, and the introduction of IAC effectively reduced the occurrence of false negatives. Compared to the RT-PCR, RAA results with DNA extraction obtained 100% sensitivity and specificity, and the RAA results with heat-treated DNA showed 85.96% sensitivity and 100% specificity.

Conclusion: With the advantages of 45 min turn-around time and simple steps of DNA purification, this assay could become a useful diagnostic tool for *Bordetella pertussis* detection and is potentially suitable for point-of-care identification to guide prompt clinical treatment.

Abbreviations: RAA, recombinase aided amplification; IAC, internal amplification control; RT-PCR, real-time PCR; LAMP, loop-mediated isothermal amplification; SSB, single-stranded DNA binding; THF, tetrahydrofuran; LFD, lateral flow dipstick; NC, negative control.

* Corresponding authors at: NHC Key Laboratory of Medical Virology and Viral Diseases, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155, Changbai Street, Changping District, Beijing 102206, China
** Corresponding author at: Hebei General Hospital, Shijiazhuang, 050051, Hebei, China.
E-mail addresses: hebmuzhangruiqing@163.com (R.-q. Zhang), 13832179762@139.com (G.-x. Li), zhaoyanghefeng@126.com (X.-n. Li), x616815@126.com (X.-x. Shen), gaoyuan@icdc.cn (Y. Gao), luka_wl@163.com (L. Wang), hugengkun@163.com (T. Fan), 15227821073@163.com (Q.-x. Duan), 462405430@qq.com (Y.-k. Wang), wangji19871229@sina.com (J. Wang), 15131129999@139.com (Z.-s. Feng), maxj@ivdc.chinacdc.cn (X.-j. Ma).

[1] Rui-qing Zhang and Gui-xia Li contributed equally to the article.

https://doi.org/10.1016/j.ijid.2019.06.028

Introduction

Pertussis is a highly transmissible acute respiratory infection caused by the bacterial pathogen *Bordetella pertussis* (Kilgore et al., 2016). In recent years, several developed countries with high vaccination coverage, such as the United States, the United Kingdom, the Netherlands, and Australia, have reported that the incidence of pertussis has risen again after maintaining low levels for many years (Domenech de Cellès et al., 2016; Rohani and Drake, 2011). Therefore, it is very important to establish accurate and rapid laboratory diagnostic methods.

Pertussis could be diagnosed by culture and serological methods. Bacteriological culture of *B. pertussis* is the gold standard diagnostic method (Dragsted et al., 2004; Tilley et al., 2000; van der Zee et al., 2015). However, culture takes about 7–10 days and has an overall 50% sensitivity (Cherry et al., 2012; Granström et al., 1991). Similarly, the Pertussis Toxin antibody detection method is not suitable for early diagnosis of disease and is also affected by vaccination, leading to false-positive result. A series of nucleic acid amplification-based assays for detecting *B. pertussis* were reported, including real-time PCR (RT-PCR) (Anderson et al., 2003), nested PCR (Bäckman et al., 1994), multiplex real-time PCR (Qin et al., 2007; Tatti et al., 2011) and loop-mediated isothermal amplification (LAMP) (Brotons et al., 2016; Kamachi et al., 2006; Nakamura et al., 2009). PCR is regarded as the gold standard in the field of molecular diagnosis, but it requires expensive equipment and professional operation. LAMP has easy operation without the need for a thermal cycles system, however, designing four or six different primers for this method is complex.

Recently, Recombinase Aided Amplification (RAA) was reported as a novel method to detect viral and bacterial pathogens (Chen et al., 2018; Shen et al., 2019; Yan et al., 2018; Zhang et al., 2017). The RAA system uses a recombinase obtained from *Escherichia coli*, which can bind tightly to the primer DNA to form a complex of the enzyme and the primer. When the primer searches for a sequence completely complementary to the template DNA, strand replacement occurs between the primers and the template with the single-stranded DNA binding (SSB) and DNA polymerase, and the new DNA fragment could be amplified rapidly *in vitro*. A specific fluorescent probe is added to the basic amplification to realize RAA real-time monitoring, and a tetrahydrofuran (THF) spacer flanked by a reporter fluorophore and a quenching fluorophore is included. When the probe binds to the target DNA, the THF position is recognized and cleaved by an Exo nuclease III only, then the fluorophore and the quencher are separated, which results in the releasing of fluorescence signal (Rong et al., 2010). The whole reaction is simple and rapid under isothermal conditions (25–45 °C) in less than 30 min. Because RAA does not require high temperature circulation, it is especially suitable for point-of-care identification to guide prompt clinical treatment.

In the present study, we developed an RAA method incorporating competitive internal amplification control (IAC) to detect *B. pertussis* using the DNA both by a commercial extraction kit and by boiling and compared its sensitivity and specificity with a standard RT-PCR assay (Higgins et al., 2018). As far as we know, it is the first report that applies the RAA method with competitive IAC to detect bacterial pathogens using the DNA obtained by boiling.

Materials and methods

Bacteria strains and clinical samples

A test panel of bacterial reference strains was used to evaluate the specificity of the RAA assay in this study, including *Bordetella pertussis* ATCC 9797, *Bordetella bronchiseptica* ATCC 10580, *Bordetella parapertussis* ATCC 15311, *Bordetella holmesii* ATCC 51541, *Corynebacterium diphtheriae* ATCC 19409, *Streptococcus pneumoniae* ATCC 6305, *Haemophilus Influenzae* ATCC 10211 and *Neisseria meningitidis* ATCC 35561, which are supplied by National State Key Laboratory for Infectious Disease Prevention and Control, Chinese Center for Disease Control and Prevention.

According to current criteria (Cherry et al., 2012), pertussis cases confirmed as patients should meet the clinical case definition, which defines a cough lasting more than two weeks with an additional symptom such as paroxysms of coughing, inspiratory “whoop”, post-tussive vomiting or apnea (for infants <1 year of age only), and meets laboratory criteria for *B. pertussis* diagnosis including isolation of *B. pertussis* from a clinical specimen or positive PCR result for pertussis. From August 2018 to March 2019, 115 clinical samples (nasopharyngeal swabs or aspirates) were collected from 115 patients with clinical suspected pertussis and admitted to the children’s hospital of Hebei, China. Meanwhile, we collected general information and vaccination history for all cases. A total volume of 0.5 mL respiratory secretions was collected in 3.5 mL transport medium and stored at −80 °C.

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China. Children’s parents were apprised of the study’s purpose and of their right to keep information.

DNA extraction and heat treatment

Bacterial load was measured by making 10-fold serial dilutions of an original suspension of *B. pertussis* (OD_{600} 1.49) and by plating in Regan-lowe medium. Plates were incubated at 37 °C in the constant temperature incubator (Luxi, Beijing, China) and were used for colony counting after 3 days. Genomic DNA extraction from all serial dilutions was performed by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Total DNA was extracted from 200 μL of clinical samples and all bacterial strains using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). All of the DNA extractions were performed according to the manufacturer's protocol. The DNA was eluted in 100 μL of elution buffer and stored at −80 °C until needed. In parallel, the clinical samples were heated at 100 °C for 15 min, followed by centrifugation (14.000 rpm/1 min) to recover supernatant and were cooled to room temperature until use (Rodrigues et al., 2017).

Primer and probe design of the internally controlled RAA assay

Conserved IS481 gene is present in high copy number (about 200) in *B. pertussis* genome and is the preferred gene for pertussis molecular detection (Cox et al., 2013; Mattoo and Cherry, 2005). The forward and reverse primers and probe were designed by Amplifx software for bacterial target and the IAC probe was derived from previously published article on the establishment of a *Rose rosette virus* RAA method (Babu et al., 2017). The bacterial probe and IAC probe were labelled with FAM and HEX fluorophores respectively. All of the primers and probes were synthesized by SangonBiotech (Shanghai, China), and the sequences are outlined in Table 1.

Preparation of bacterial plasmid and IAC plasmid

A 420 bp fragment (nt3356943-3357362, GenBank accession no. CP014211.1) of the IS481 gene of *pertussis,* covering primers and probe sequences described above, was cloned into the pUC57 Vector to construct the Bacterial plasmid. The IAC plasmid was similarly constructed but replacing the bacterial probe with the IAC probe based on the Bacterial plasmid (Figure 1). The Bacterial plasmid and

Table 1
Primer and probe sequences used for RAA and IS481-based RT- PCR assay.

Assay	Primers and Probes	Sequence 5′-3′	Amplicon size
RAA	Forward	AAATCGCCAACCCCCCAGTTCACTCAAGGA	125 bp
	Reverse	GCACACAAACTTGATGGGCGATCAATTGCT	
	Probe[a]	TGAACACCCATAAGCATGCCCGATTGACCT[FAM-dT](dSpacer)C[BHQ-dT]ACGTCGACTCGAAA(C3 Spacer)	
Internal Amplification Control	Probe[b]	GTAAGGTGCTAGACTAAAATTGTTGGGACTT[HEX-dT]G(dSpacer)A[BHQ-dT]CTCTGAAGTAAAAGG(C3 Spacer)	
IS481-based Real-Time PCR	Forward	CAAGGCCGAACGCTTCAT	66 bp
	Reverse	GAGTTCTGGTAGGTAGGTGTGAGCGTAA	
	Probe	FAM-CAGTCGGCCTTGCGTGAGTGGG-BHQ1	

[a] Probe modifications: FAM, 6-carboxyfuorescein; THF, tetrahydrofuran; BHQ, black hole quencher; C3Spacer, 3′phosphate blocker
[b] Probe modifications: HEX, 5-hexachlorofuorescein; THF, tetrahydrofuran; BHQ, black hole quencher; C3Spacer, 3′phosphate blocker

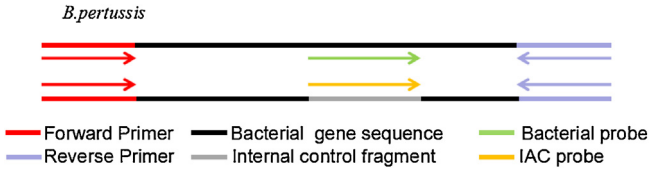

Figure 1. Schematic diagram of the internally controlled RAA assay for detection of *B. pertussis*.

IAC plasmid were quantified using a Qubit® dsDNA BR Assay Kits (Life technologies Invitrogen). Plasmid copy number was calculated using the following formula: Plasmid copy number ce:hsp sp="0.25"/>=(copy number/μL) = {[6.02 × 10^{23} × plasmid concentration (ng/μL) × 10^{-9}]}/[Plasmid length × 660]. Ten-fold dilutions of Bacterial plasmid ranging from 10^4 to 10^0 copies/μL were used as standards for the sensitivity analysis of Internally controlled RAA assay. IAC plasmid was diluted to 1000, 100 and 50 copies/μL in order to obtain the optimum concentration.

Analytical sensitivity and specificity of the internally controlled RAA assay

The Internally controlled RAA assay was performed in a 50 μL volume using RAA exo kit (Qitian, Jiangsu, China). The reaction components included DNA templates (extracted or plasmid when appropriate), 25 μL of rehydration buffer, 2.1 μL of each primer (10 uM), 0.6 μL of bacterial probe (10 uM), 0.6 μL of IAC probe (10 uM), 1 μL IAC plasmid (1000, 100 and 50 copies/μL) and RNAse-free water. Then, 47.5 μL of the master mix was added to each reaction tube containing a dried enzyme pellet, and 2.5 μL of 280 mM magnesium acetate was pipetted into the tube lids. The lids were carefully closed, and the tubes were vortexed and centrifuged using the equipment QT-RAA-B6100 (Qitian, Jiangsu, China) at 39.0 °C for 4 min. The tubes were then performed by an RAA fluorescence detection device QT-RAA-F1620 (Qitian, Jiangsu, China) at 39 °C for 30 min. Negative control (nuclease-free water) was included in each run. Positive results were determined by the equipment QT-RAA-F1620 by setting the slope K value to be greater than or equal to 20 and was simultaneously recorded in the FAM and HEX detection channels. The optimum concentration of the IAC plasmid and the sensitivity were evaluated by testing the bacterial plasmid ranging from 10^4 to 10^0 copies/μL and *B. pertussis* strain between 10^5 to 10^0 CFU/ml in the presence of various IAC copy concentrations, 1000, 100 and 50 copies/μL.

The analytical specificity of the primers and probes was performed using extracted DNA of a test panel of bacterial strains as described above.

Clinical evaluation of the internally controlled RAA assay and the statistical analysis

A total of 115 samples with clinical suspected pertussis were detected by the internally controlled RAA assay using 2 μL of DNA extracted or 4 μL heat-treated mixture as templates. A previously reported IS481-based real-time PCR was also performed in parallel (Tatti et al., 2011). A negative control (nuclease-free water) was included in each run.

IBM SPSS Statistics, version 21 (IBM Corporation, NY, USA) was used to perform all of the statistical analysis. The results of clinical detection by IS481-based RT-PCR assay and RAA assay were analyzed using Kappa and McNemar's tests, and a value of $P < 0.05$ was considered statistically significant.

Results

The Analytical specificity and sensitivity of internally controlled RAA assay

The RAA results of Strains of *B. pertussis* and *B. holmesii* were positive. Meanwhile, the remaining other strains and the negative control were determined as negative. The method established in this study showed 100% specificity for Bordetella bacillus. No cross reaction with other strains was observed.

The optimum concentration of the IAC plasmid was determined to be 100 copies for each internally controlled RAA assay (Figure 2). A panel of serial 10-fold dilutions of the bacterial plasmid from 10^4 to 10^1 copies per reaction and *B. pertussis* strain from 10^5 to 10^1 CFU/ml were tested positive to ascertain the endpoint dilution. As shown in Figure 2, the sensitivity of the internally controlled RAA assay was 10^1 copies or 10 CFU/ml per reaction, and the negative control (nuclease-free water) was observed only in the HEX channel.

Evaluation of the internally controlled assay using clinical samples and comparison with RT-PCR

The internally controlled RAA was tested to demonstrate the clinical performance using extracted DNA and heat-treated specimens. For comparison, IS481-based RT-PCR was also performed in parallel. As shown in Table 2, a total of 115 clinical samples were tested in parallel, of which 57 were positive by IS481-based RT-PCR assay and the internally controlled RAA assay with DNA extraction. In comparison to IS481-based RT-PCR, RAA correctly identified and differentiated all 57 positive samples (C_T value of less than 35) with 100% sensitivity and specificity. The results were further analyzed

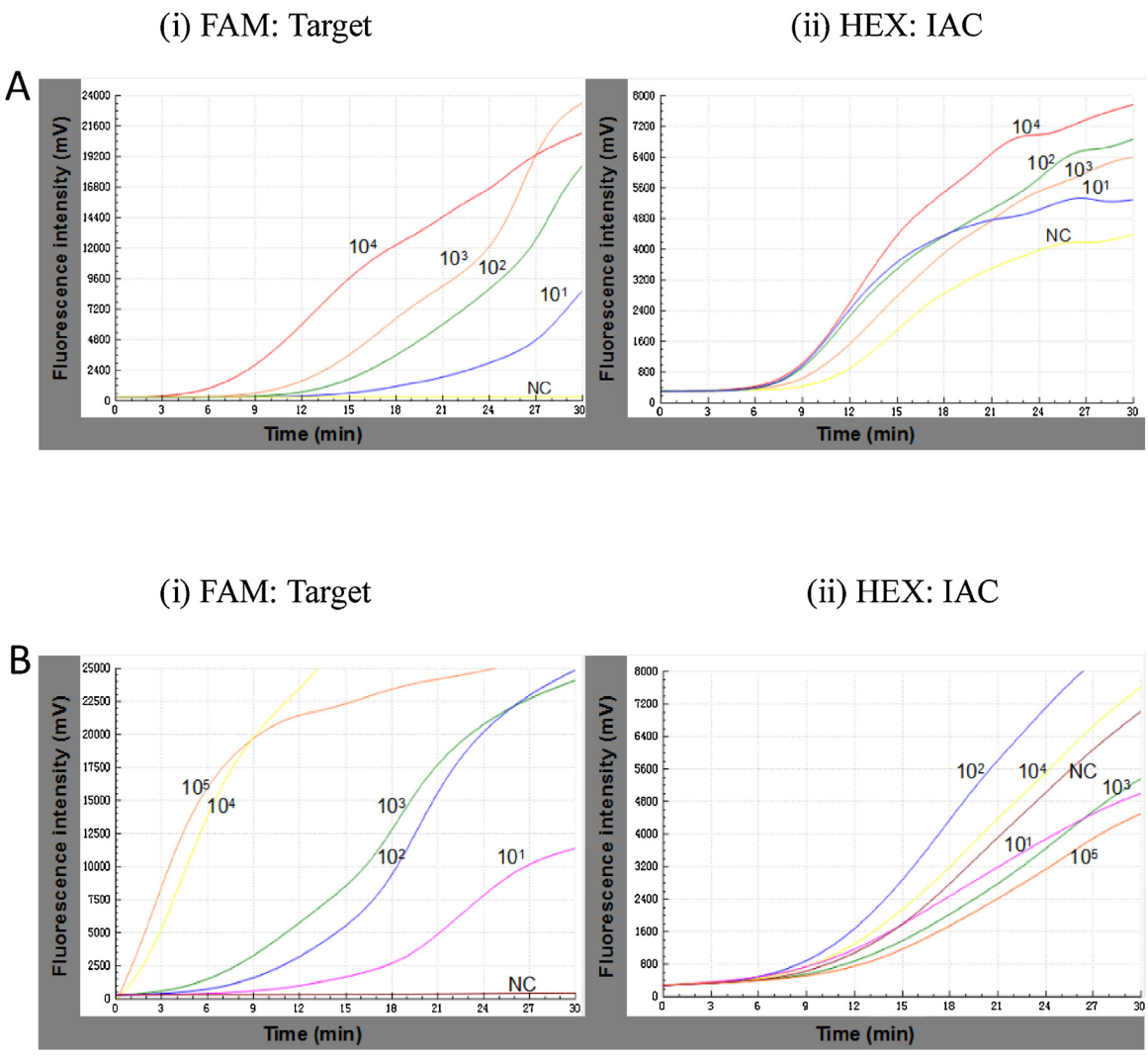

Figure 2. Sensitivity of internally controlled RAA assays for *B. pertussis* in the presence of 100 copies IAC. A panel of serial 10-fold dilutions of (A) Bacterial plasmid from 10^4 to 10^1 copies per reaction and (B) *B. pertussis* strain from 10^5 to 10^1 CFU/ml were tested. (i): The NC reaction was negative in the FAM channel. (ii): The NC reaction was observed only in the HEX channel where IAC template was detected in the absence of a bacterial target. NC, negative control.

Table 2
The clinical performance of the RAA with heat treatment or with DNA extraction compared with IS481-based RT- PCR as the reference method

		IS481-based Real-Time PCR		Performance characteristics		
		Positive	Negative	Sensitivity (%)	Specificity (%)	Kappa
RAA (heat-treatment)	Positive	49	0	85.96	100	0.861
	Negative	8	58			
	Total (n = 115)	57	58			
RAA (DNA extraction)	Positive	57	0	100	100	1.000
	Negative	0	58			
	Total (n = 115)	57	58			

statistically using Kappa and McNemar's tests. The results showed no significant differences between the RAA assay and the IS481-based real-time PCR assay according to the Kappa coefficient, with Kappa values of 1.000 ($P < 0.001$).

Meanwhile, eight samples (C_T value ranging from 32.89 to 35) by IS481 real-time PCR were negative by the internally controlled RAA assay using the DNA obtained by boiling. Diagnostic sensitivity and specificity of RAA assay compared to RT-PCR were 85.96% and 100%, respectively.

Characterization of the clinical samples

Pertussis was detected in 57 (49.57%) of 115 clinical samples with suspected pertussis. Of those 33 (57.89%) were male and 24 (42.11%) were female. Ages ranged from 43 days to 10 years old, and 35 (61.40%) were under 6 months. Among them, 18 cases were 1–3 months old, 17 cases were 4–6 months old, 19 cases were 7–36 months old, and 3 cases were over 3 years old. Additionally, 21 cases (36.73%) were not vaccinated, 22 cases (38.78%) had not

completed the vaccine process, and 14 cases (24.49%) were completely vaccinated.

Discussion

In this work, we developed an internally controlled RAA assay for rapid, simple and sensitive detection of *Bordetella pertussis.* We evaluated the diagnostic performance of the internally controlled RAA assay for detection of IS481 of *B. pertussis* using 115 clinical samples, of which 57 were positive by IS481-based RT-PCR assay and the RAA assay with DNA extraction, demonstrating 100% sensitivity and specificity. Meanwhile, 49 samples (C_T value ranging from 17 to 32.89) by IS481 real-time PCR were positive by the RAA assay using the DNA obtained by boiling with 85.96% sensitivity and 100% specificity. The preliminary evaluation of diagnostic performance is very encouraging, revealing fair agreement between the RAA assay and RT- PCR, but more comprehensive evaluation of the internally controlled RAA assay using larger sets of clinical samples and the DNA obtained by boiling will be needed in the future. To our knowledge, this article is the first report to use the RAA method for the diagnosis of *B. pertussis* infection.

RAA is a low-cost, simple-to-use, ideal for point-of-care diagnostics technology for the detection of *B. pertussis*. Traditional PCR technology requires a thermal cycle process, including denaturation, annealing and extension of 40 cycles and about 3 h. Besides, the application of PCR outside the laboratory is difficult. Thermostatic nucleic acid amplification appears to overcome this shortcoming of traditional PCR, such as LAMP and RAA technology (Rong et al., 2010; Torkaman et al., 2015). Particularly, the RAA in this study could produce a positive signal at 37 °C for 30 min in portable equipment. The RAA operation is simple and the cost of RAA is about half that of LAMP. Moreover, the RAA kits containing the lyophilized reaction pellets are very convenient for transport because lyophilized reagents are stable at room temperature. The amplification products obtained by the LAMP method are fragments of different sizes, which cannot be directly cloned and sequenced. This is the biggest limitation of the LAMP method. In the case of RAA, amplified products by sequencing can be used to confirm false positives. In summary, RAA is more suitable for the field detection of *B. pertussis.*

In order to reduce false-negative results, we added competitive internal control, consisting of an IAC plasmid and IAC probe. After IAC plasmid was determined to be 100 copies, the sensitivity of the internally controlled RAA assay was 10^1 copies/μL or 10 CFU/ml per reaction. Because the Bacterial probe and IAC probe were labelled with FAM and HEX fluorophores, respectively, and high concentration of specimens might inhibit the amplification of internal control, the specimen is considered as positive as long as the FAM channel generates a positive signal regardless of the signal in HEX channel. However, if the FAM and HEX channels produce negative signal, the result is considered false-negative, suggesting operation error, or poor enzyme activity, or the reaction was inhibited. The IAC was not detected during the evaluation of clinical samples with the Ct values ranging from 17 to 20 by RT- PCR as we only make sure amplification of the IAC in situations in which the targets are not being detected (Hoorfar et al., 2004). Therefore, the addition of competitive IAC can reveal failure of the RAA, warranting that a negative response was indeed due to the absence of the target sequence in the reaction. Thus, RAA assay incorporating competitive IAC diagnosis of *B. pertussis* has a good application prospect in the hospital, and it provides evidence for clinicians to adjust the treatment plan, avoids abuse of antibiotics and save medical resources and costs.

In our study, we successfully performed the internally controlled RAA assay using the DNA obtained by boiling. Most of the heated-treated samples (100 °C for 15 min) were positive by the internally controlled RAA assay, and only eight specimens with low concentration (C_T value ranging from 32.89 to 35) were negative. The result indicated that simple-pretreatment samples can be applied directly as templates to the RAA system. This assay simplified the DNA extraction step, and saved time and money. In addition, the RAA device is portable, making this assay more suitable for applications in resource-poor settings. Next, we plan to combine this assay with lateral flow dipstick (LFD) in order to perform a visible and equipment-free LFD RAA assay (Liu et al., 2018).

Analytical specificity of the internally controlled RAA assay failed to differentiate *B. holmesii* from *B. pertussis*. Like *B. pertussis*, *B. holmesii* contains IS481 elements (8–10 copies per genome) and could lead to a false-positive result (Loeffelholz et al., 2000; Reischl et al., 2001). Nevertheless, as conserved IS481 gene is present in a high copy number in *B. pertussis* genome, molecular methods based on IS481 sequence are commonly recognized to be highly sensitive (Anderson et al., 2003; Dragsted et al., 2004; Cox et al., 2013), Other genes have been designed for molecular diagnosis targets for pertussis, including IS1002, PT and BP485 (Kamachi et al., 2006; Liu et al., 2015; Roorda et al., 2011), but they have not been widely used as the result of lower sensitivity than IS481 target. Since our main focus was to develop an assay for rapid preliminary screening diagnosis in a clinical setting, sensitivity is of the most concern to reduce or avoid false-negatives while providing rapid results to ensure appropriate treatment and prevention of further transmission (Tatti et al., 2011). Considering that the higher sensitivity of the IS481 target than of other targets and potential for cross-reaction is limited, it is still suitable for using IS481 to detect pertussis (Cox et al., 2013). Future optimizations of the assay design could include the combination of two genes in multiplex RAA to improve the specificity of the test.

At present, the recommended immunization procedure of pertussis vaccine in China is 3 doses of basic immunization at the age of 3, 4 and 5 months, and 1 dose of strengthening immunization at the age of 18-24 months. This vaccination program has a better protective effect on preschool children and school-age children who have completed basic immunization or have completed strengthening immunization recently (WHO, 2016). This study showed that the age of children with pertussis is still mainly young infants aged 1–6 months, and there are few children over 3 years old (Takahashi et al., 2003). Among 57 positive samples, 43 (75.45%) are not vaccinated or have not completed the whole vaccination, suggesting that the young children who have not been vaccinated or have not completed the whole vaccination is a high-risk group of pertussis in children (McNabb et al., 2008). The most effective measure to prevent and control pertussis is still to receive vaccine. However, due to changes in the current pattern of pertussis, the onset of the group has shifted to adults and adolescents with atypical clinical manifestations (Forsyth et al., 2015). Therefore, RAA detection of pertussis DNA provides evidence for early diagnosis of whooping cough (pertussis), effect observation after vaccination and epidemiological study.

In conclusion, we have developed and validated an RAA assay incorporating competitive internal control for diagnosis of *B. pertussis* in clinical samples using the DNA obtained by boiling. With the advantages of 45 min turn-around time and simple steps of DNA purification, RAA assay could potentially become a prospective diagnostic tool for point-of-care *B. pertussis* identification and clinical application.

Ethics approval and consent to participate

All aspects of the study were performed in accordance with national ethics regulations and approved by the Institutional

Review Boards of National Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of China and the Ethics Committee of Children's hospital of Hebei Province, china. The written informed consents were obtained from the children's parents after informed them the use of data for analysis and using the results for improving patient care activities and without disclosing their names or identity.

Consent for publication

Not applicable.

Funding

This work was supported by grants from the China Mega-Project for Infectious Disease (2018ZX10711001, 2017ZX10104001 and 2017ZX10302301-004-002). A Chinese patent (KHP191113234.5) is pending.

Conflict of interests

All the authors approved the final manuscript and they have no conflict of interest to declare.

Authors' contributions

Xue-jun Ma, Zhi-shan Feng, Ji Wang and Rui-qing Zhang designed the study; Rui-qing Zhang and Gui-xia Li performed the experiments; Rui-qing Zhang, Ji Wang and Xue-jun Ma writing; Wang Le, Ya-kun Wang and Yuan Gao, sample collection; Tao Fan and Qing-xia Duan, data analysis. All authors provided critical review and approved the manuscript.

Acknowledgments

We acknowledge the children's hospital of Hebei Province, China for providing clinical specimen and the National State Key Laboratory for Infectious Disease Prevention and Control, Chinese Center for Disease Control and Prevention for providing bacterial reference strains.

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