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A lateral flow dipstick combined with reverse transcription recombinase polymerase amplification for rapid and visual detection of the bovine respirovirus 3

Guimin Zhao\textsuperscript{a,b}, Hongmei Wang\textsuperscript{b}, Peili Hou\textsuperscript{b}, Xianzhu Xia\textsuperscript{a,c,*}, Hongbin He\textsuperscript{b,**}

\textsuperscript{a} College of Animal Science and Technology, Shihezi University, Shihezi, 832003, China
\textsuperscript{b} Key Laboratory of Animal Assistant Biology of Shandong, Ruminant Disease Research Center, College of Life Science, Shandong Normal University, Shandong Province, China
\textsuperscript{c} Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary, Academy of Military Medical Sciences, Changchun, 130122, China

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\textbf{A B S T R A C T}

Bovine respirovirus 3 also known as Bovine parainfluenza virus type 3 (BPIV3) is one of the most important viral respiratory agents of both young and adult cattle. Rapid diagnosis could contribute greatly in containing epidemics and thus avoid economic losses. However, the lack of robust isothermal visual method poses difficulty. In this study, a novel isothermal assay for detecting BPIV3 was established. The method includes a lateral flow dipstick (LFD) assay combined with reverse transcription recombinase polymerase amplification (RT-RPA). First, the analytical sensitivity and specificity of BPIV3 LFD RT-RPA were tested. The LFD RT-RPA assay has a detection limit of up to 100 copies per reaction in 30 min at 38 °C. Then the performance of LFD RT-RPA was evaluated using 95 clinical samples. Compared to qPCR, the LFD RT-RPA assay showed a clinical sensitivity of 94.74%, a clinical specificity of 96.05% and 0.8734 kappa coefficient. These results have demonstrated the efficiency and effectiveness of the method to be developed into a point of care protocol for the diagnosis of BPIV3.

1. Introduction

Bovine respirovirus 3 also known as Bovine parainfluenza virus type 3 (BPIV3) is a member of the \textit{Paramyxoviridae}, genus \textit{Respirovirus}. BPIV3 is one of the most important viral respiratory agents of both young and adult cattle \cite{1}. While most acute infections are subclinical, they can cause respiratory disease characterized by cough, fever and nasal discharge \cite{2}. In some instances where animals are also subjected to high stress, resulting in severe bronchopneumonia from secondary bacterial infections \cite{3}. A variety of factors, such as environmental temperature, transportation, hygiene, stocking density, co-mingling and host immune status, can contribute to increased susceptibility to secondary bacterial infection and severity of clinical disease \cite{3}. BPIV3 has been associated with the bovine respiratory disease complex (BRDC) \cite{4,5}, which is a major health problem of cattle worldwide. Thus, early detection of clinical infection is important as it can facilitate more rapid implementation of rigorous controls, which can result in reduced health care costs and improved cure rates.

The common and widely accepted molecular technique for detecting BPIV3 is reverse transcriptase polymerase chain reaction (RT-PCR) or quantitative RT-PCR (RT-qPCR) \cite{6,7}. However, this method requires thermal cycling equipment and the settings of professional diagnostic laboratory. Moreover, most nucleic acid amplification technologies assays, including many isothermal amplification methods, require power-dependent instrumentation for incubation \cite{8}. With a reaction time of 1–2 h, these can also be fairly time-consuming. Recently, recombinase polymerase amplification (RPA), a novel isothermal technique, has emerged as an ideal molecular technology for rapid and economical diagnostics \cite{9}. With the development of nucleic acid purification technology independent of a laboratory, a lateral flow dipstick (LFD) combined with a RT-RPA assay is especially suitable for on-site diagnosis of clinical specimens. The commercial reverse transcriptase kits (PrimeScript RT Master Mix; TaKaRa, Dalian, China) incubate the samples for 15 min at 37 °C. The RPA tolerates temperatures ranging from 30 to 42 °C without losing reaction efficiency and has been so far already successfully used to construct rapid detection of

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many pathogens [8,10–13].

In this study, we established a LFD RT-RPA assay for detection of BPIV3 using oligonucleotides targeting a conserved region of HN gene [6,14]. Furthermore, we evaluated the detection sensitivity, specificity and performance of LFD RT-RPA assay when compared with the qPCR or qPCR methods to assess its performance and reliability as a potential point-of-care field test.

2. Materials and methods

2.1. Viruses and clinical specimens

Bovine respiratory virus 3 (Bovine parainfluenza virus type 3, BPIV3)/BN-1 strain, Bovine alphaherpesvirus 1 (infectious bovine rhinotracheitis virus, IBR)/BarthaNu/67 strain, Pestivirus A (bovine viral diarrhea virus 1, BVDV1)/NADL strain were preserved in our laboratory, and the viruses were cultured following previously published procedure in our laboratory [11]. Other bovine virus strains used in this study were provided by full-length cloned cDNA: entire genome sequence of bovine orthopneumovirus (bovine respiratory syncytial virus, BRSV)/A51908strain, bovine fever hemorrhovirus (bovine ephemeral fever virus, BEFV)/(Accession number: JX234571.1) [15], Betacoronavirus 1 (bovine coronavirus, BCoV)/ENT strain (Accession number: NC_003045.1), respectively. The cDNA of Indiana vesiculovirus (vesicular stomatitis virus, VSV) was kindly provided by Wenqi He (Jilin University, China).

A total of 95 clinical specimens (62 nasal swabs and 33 tissue specimens) were collected between January 2017 and February 2018 from 62 cattle from 17 different dairy farms suspected to be infected with bovine respiratory disease (BRD). The 17 farms were located in seventeen distinct geographic regions [16,17] of Shandong province, China. A respiratory disease score was assigned based on rectal temperature, the character of nasal discharge, eye or ear appearance, and presence of a cough based on the method by Lago et al. (2006) [18]. Cattle with a score of 6 or higher had at least two clinical signs of respiratory disease and were thus considered sick, and the 62 nasal swab samples taken from all 17 dairy farms in which case suggests after BRD. 33 fresh lung samples were sampled from postmortem calf with BRD.

2.2. Isolation of DNA/RNA, cDNA synthesis

DNA of IBRV and RNA of BVDV, BPIV3 was extracted following a previously published procedure in our laboratory [19,12]. The DNA/RNA was eluted in 50 μL of nucleic-acid-free water. The extracted RNA was used as template for cDNA synthesis using reverse transcription with random primers according to the instructions of the PrimeScript RT Master Mix (TaKaRa, Dalian, China). All templates were stored at −70 °C until further needed.

2.3. Generation of RNA molecular standard

The BPIV3 HN gene (1719 bp) was cloned into pGEM-T Easy vector (Promega, USA). RNA molecular standard was prepared as previously described [20] with some modifications. In brief, the recombinant plasmid was linearized by Nde I (Thermo Fisher Scientific, USA), and purified using the MiniBEST DNA Fragment Purification Kit (TaKaRa, Dalian, China). A total of 1 μg of linearized product was used for in vitro transcription (T7 Ribomax Large Scale RNA Production System, Promega, USA) following the manufacturer’s instructions. In vitro transcribed BPIV3 RNA was digested with the supplied RNase-free DNase and purified. The purified RNA was quantified with the Quant-itTM Ribogreen RNA Assay Kit (Thermo Fisher Scientific, USA) in accordance with the manufacturer’s instructions. The copy number of RNA molecules was calculated by the following formula: Amount (copies/µL) = [RNA concentration (g/µL)/(transcript length in nucleotides × 340)] × 6.02 × 10²³.

2.4. Design of RPA primers and LF probe

By alignment analysis of the HN gene with the data from NCBI/GenBank (DB4095.1; AF178655.1; NC_002161.1; JQ063064.1; EU1277658.1; HQ530153.1; JX969001.1; AB770484.1; LC000638.1), six combinations of candidate primers (3 forward and 2 reverse) and one TwistAmp LF-probe were designed according to RPA operating instructions of TwistDx (TwistDx, Cambridge, UK), and synthesized by Sangon Biotech (Shanghai, China). In summary, the TwistAmp LF Probe constitutes of an oligonucleotide backbone, which includes a 5′-anti-genic label FAM group, an internal abasic nucleotide analogue “dSpacer” and a 3′-polymerase extension-blocking group C3-spacer. One amplification primer opposing TwistAmp LF Probe is labeled with Biotin at its 5′ end in order for the dual-labeled amplicon to be detected simultaneously. Oligonucleotide sequences of RPA primers and LF probes used in the study are listed in Table 1 and Fig. S1.

2.5. BPIV3 LFD RT-RPA assays

Reaction of RPA and visualization of RPA amplicons were carried out following previously published procedure in our laboratory [12,13]. The BPIV3 RPA was performed in a 50 μL final reaction volume according to instructions outlined in the TwistAmp info kit (TwistDX, Cambridge, UK). In brief, the rehydration solution contained 1 μL DNA template, 2.1 μL (10 μM) forward primers and reverse primers, respectively, 0.6 μL (10 μM) TwistAmp LF Probe. All test samples were incubated for a prototypical 25 min at 38 °C. The tubes were incubated at 38 °C in an incubator block for 4 min. As recommended, samples were blended top down and bottom up 6–8 times after 4 min incubation, and an additional incubation was continued for 21 min. Then RPA products were purified using MiniBEST DNA fragment purification kit (TaKaRa, Dalian, China). A 2% agarose-gel electrophoresis was performed to determine the size of the products. Visualization of the amplicons was carried out using a LFD tool kit (HybriDetect, Milenia Biotec GmbH, Germany): 2 μL of RPA product and 98 μL HybriDetect assay buffer were blended in a 200 μL centrifuge tube, then the dipstick was put directly into the buffer and maintained at an upright position for 5 min under room temperature.

To determine the optimal reaction condition, the preferred temperature (30 °C, 34 °C, 38 °C, 42 °C, 45 °C and 48 °C) and time (5 min,
analytical speciﬁcity of the assay

To assess the detection limit of BPIV3 genomic copies, the in vitro transcribed RNA was diluted in a 10-fold serial dilution manner to achieve RNA concentrations ranging from $10^7$ to $10^1$ copies/μL. One μL of each dilution was ampliﬁed by RT-RPA reactions within the same sample run to determine the analytical sensitivity of the assay. The analytical speciﬁcity of the assay was assessed among other viral pathogens of cattle. DNA of IBRV, RNA of BVDV and BPIV-3 were prepared from cell culture supernatant, and cDNA of BVDV, BPIV-3 was prepared as above mentioned. Clone of full-length genome of BEFV, BRSV, BcCoV and VSV were supplied as templates in the LFD RT-RPA reaction. Additionally, $10^4$ copies RNA molecular standard per reaction and BPIV3-free samples was used as a positive control and a negative control respectively.

2.7. Clinical specimen preparation

BPIV3-free nasal swab specimens were obtained from healthy calves for negative control. A total of 95 clinical specimens including 62 clinical samples and 33 lung tissue specimens were collected from suspected dairy cattle cases of BPIV3 infections in Shandong Province, China. Details of clinical specimen were listed in Table 2. The extraction of RNA from clinical specimens and cDNA synthesis was prepared following previously published procedure in our laboratory [12]. The volume of 1 μL of cDNA extracted from each specimen was used as a template in the LFD RT-RPA reactions.

2.8. Real-time qPCR and RT-PCR for ampliﬁcation of BPIV3

The RT-qPCR assay was performed on a LightCycler 480 II (Roche, Germany) using the Probe qPCR Mix (TakaRa, Dalin, China) with the primers and probe described by Horwood and Mahony [21]. Brieﬂy, the primer pairs for real-time qPCR used for ampliﬁcation of BPIV3 were BPIV3-F: 5′-TGCTTCATAGATAGGACAAAATT-3′, BPIV3-R: 5′-GCAATGATAACAATGCCATGGA-3′, and probe: 5′-FAM-ACAGCAATGGATCAATAA-MGB-BHQ2-3′. The reaction was prepared as a 20 μL reaction volume containing 1 μL of the cDNA template, 200 nM of each primer, 200 nM of each probe, 2 × Probe qPCR Mix, and sterile DNease-free water. The following thermal cycling parameters: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, and 60 °C for 7 s followed by a final cooling step at 40 °C for 30 s. The results were analyzed by gene scanning software version 1.5 (Roche, Germany). The assessment of clinical samples was also carried out by RT-PCR assay following previously described by our laboratory [12]. Two methods were operated with the same amount of template.

3. Results and discussion

Firstly, the analytical speciﬁcity of RPA primers and LF-probes was performed by agarose-gel electrophoresis consulting recent studies [12]. We've especially noticed that when LF-probe is cut by nfo nuclease at the dSpacer position, the probe will be transformed into a

Table 2

| Samples          | Number of samples | RT-PCR Positive | RT-PCR Negative | Real-time qPCR Positive | Real-time qPCR Negative | LFD RT-RPA Positive | LFD RT-RPA Negative |
|------------------|-------------------|-----------------|-----------------|-------------------------|-------------------------|---------------------|---------------------|
| nasal swabs      | 62                | 9               | 53              | 11                      | 51                      | 13                  | 49                  |
| fresh lungs      | 33                | 6               | 27              | 8                       | 25                      | 8                   | 25                  |
| Total            | 95                | 15              | 80              | 19                      | 76                      | 21                  | 74                  |
Conclusions

A LFD RT-RPA assay has been developed for the rapid detection of BPIV3 and by comparison with a previously published qPCR its performance was shown to be comparable. The LFD RT-RPA could be potential applicability in the field where simple and rapid diagnosis of BPIV3 is required, especially in the resource-limited settings.

Ethics approval and consent to participate

Experimental protocols for obtaining cattle clinical samples used in this study were carried out in strict accordance with the Chinese Regulations of Laboratory Animals (Ministry of Science and Technology of People’s Republic of China, 20110108), and the animal study...
Table 3
Sensitivity, specificity, predictive value, and kappa value of LFD RT-RPA and RT-PCR or real-time qPCR methods for diagnosing BPIV3 infection.

|         | RT-PCR                  | Real-time qPCR                  |
|---------|-------------------------|---------------------------------|
|         | P          | N        | T      | P    | N    | T    |
| LFD RT-RPA | P 15   | 6        | 21     | 18   | 3    | 21   |
|         | N 0      | 74       | 74     | 1    | 73   | 74   |
|         | T 15     | 80       | 95     | 19   | 76   | 95   |
|         | Se:100%  | Sp:92.50% | K:0.7957 | Se:94.74% | Sp:96.05% | K:0.8734 |
|         | PPV:71.43% | NPV: 100% |

Note: P, Positive; N, Negative; T, Total; Se, Sensitivity; Sp: Specificity; K: Kappa value; PPV: Positive predictive value; NPV: negative predictive value.

The proposal was approved by Shandong Normal University Animal Care and Use Committee (approval No. 20160901).

Conflicts of interest
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors’ contributions
HBH and XXZ conceived and designed the experiments. ZGM and WHM performed the experiments. ZGM, WHM, and HPL analyzed the data. ZGM and HBH wrote the paper. All the authors read and approved the final manuscript.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at https://doi.org/10.1016/j.mcp.2018.08.004.

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