A one-step reverse transcription loop-mediated isothermal amplification for detection and discrimination of infectious bursal disease virus

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Abstract

Background: Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease in young chickens caused by infectious bursal disease virus (IBDV). It causes huge economic losses to the poultry industry. The objective of this study is to develop a loop-mediated isothermal amplification (LAMP) method for the detection and discrimination of IBDV.

Results: In this study, we applied reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect IBDV in one simple step and further identified the very virulent strain from non-vvIBDVs with a simply post-amplification restriction enzyme analysis. Based on sequence analysis, a set of two inner, two outer and two loop primers were designed to target the VP5 gene and they showed great specificity with no cross reaction to the other common avian pathogens. The detection limit determined by both color change inspection and agarose gel electrophoresis was 28 copies viral RNA, which was almost as sensitive as a real-time RT-PCR previously developed in our laboratory. We also identified a unique Tfl I restriction site located exclusively in non-vvIBDVs, so very virulent strain could be distinguished from current vaccine strains. By screening a panel of clinical specimens, results showed that this method is highly feasible in clinical settings, and it obtained results 100% correlated with real-time RT-PCR.

Conclusion: RT-LAMP is a rapid, simple and sensitive assay. In combination with the Tfl I restriction analysis, this method holds great promises not only in laboratory detection and discrimination of IBDV but also in large scale field and clinical studies.

Background

Infectious bursal disease virus (IBDV) is the etiologic agent of infectious bursal disease (IBD), an acute and highly contagious disease affecting young chickens. Characterized by immunosuppression and a high rate of mortality, this disease causes a huge economic loss to the poultry industry worldwide [1]. In recent years, IBD has rarely showed the typical clinical symptoms and become less responsive to the conventional vaccination. Very virulent IBDV (vvIBDV) causing severe mortality in chickens has become the dominant strain responsible for several disease outbreaks in China [2]. To control this disease, a sensitive, reliable, rapid and clinically feasible method for the detection of the virus and identification of the very virulent strain at early stage of infection is urgently needed.

Developed by Notomi et al., loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method with high specificity and sensitivity under isothermal condition [3]. It is also a robust method that produces a high amount of products sufficient for real time monitoring by visual inspection. In addition, RNA can be directly used as starting material by reverse transcription coupled with loop-mediated isothermal amplification (RT-LAMP) in one step [4-8], making it ideal for detection of RNA-viruses such as IBDV. Previously, in a field diagnostic testing, RT-LAMP showed great superiority over conventional RT-PCR [9,10]. More
recently, it has been successfully applied for the detection of IBDV [11,12]. However, none of these studies differentiated virus types.

In this study, we applied RT-LAMP to detect IBDV in one simple step and further identified the very virulent strain from the non-vvIBDVs with a post-amplification restriction digestion analysis. We show here that this method is very efficient and convenient compared with conventional RT-PCR and real-time RT-PCR, and also high feasible with clinical specimens.

**Methods**

**Virus strains**

IBDV Gt strain was attenuated from the vvIBDV Gx strain by continuous passage in specific-pathogen-free chicken embryos for 5 generations and in chicken embryo fibroblasts for 20 generations [13]. IBDV Gt, IBDV D78, vvIBDV Gx and chicken anemia virus (CAV) M9905 were all stock strains of our laboratory. Other avian pathogens, such as avian influenza virus (AlV) A/Chicken/Shandong/6/96 (H9N2), Newcastle disease virus (NDV) La sota, infectious bronchitis virus (IBV) F and Marek’s disease virus (MDV) CV1988 were obtained from the Harbin Veterinary Research Institute, China.

**Sequence analysis and primer design**

Sequence data for 57 IBDV isolates including vvIBDVs (Genbank accession numbers: [AF092943], [AF240686], [AF247006], [AF262030], [AF322444], [AF362776], [AF508176], [AF527039], [AF533670], [AJ318896], [AJ879932], [AY099456], [AY134874], [AY323952], [AY444873], [AY520909], [AY520910], [AY520911], [AY598356], [AY665672], [AY769978], [AY780418], [D49706], [DQ286035], [DQ927042], [EF517528]) and non-vvIBDVs (GenBank accession numbers: [AY134875], [D00867], [D00868], [DQ187988], [DQ403248], [EF418035], [EF418034], [EF418033], [M66722], [X03993], [X16107], [X84034]) were in vitro transcribed with T7 Cap-Scribe (Roche, Germany) from plasmid pcDNA3.1-GtVP5 carrying the VP5 gene of Gt strain under the control of T7 promoter. To evaluate the sensitivity of RT-LAMP, RNA standards were in vitro transcribed with T7 Cap-Scribe (Roche, Germany) from plasmid pcDNA3.1-GtVP5 carrying the VP5 gene of Gt strain under the control of T7 promoter. RNA was quantified by spectrophotometer, and then 10-fold serially diluted from 2.8 × 10⁸ copies/µL to 2.8 × 10⁵ copies/µL and used as templates for RT-LAMP. The lowest amount of RNA detectable under the conditions described above was defined as the detection limit.

**Identification of vvIBDV by Tfi I restriction fragment analysis**

RT-LAMP products were digested with Tfi I in a 20 µL reaction containing 3 µL RT-LAMP product, 1 × NEBuffer 3, 1 × BSA and 2.5 U TfiI (New England Biolabs, USA).
After incubation at 65°C for 1 hour, 10 μL aliquot was subjected to electrophoresis on 2% agarose gel and stained with ethidium bromide. The DNA band pattern was visualized with a UV transilluminator and photographed.

**Clinical specimen evaluation**

From 1999 to 2008, samples from Bursa of Fabricius in chickens exhibiting skeptical pathologic features of IBD were collected in different commercial broiler and layer farms from 11 provinces of China. All specimens were processed according to the International Cooperation with Developing Countries project method. Tissues were homogenized as described previously [2]. Viral RNA extraction, RT-LAMP, product analysis and Tfi I digestion were carried out essentially the same as described above.

**Reverse transcription**

12 μL viral RNA and the segment specific primer R (5’-CCATTGTAGCTAACATCTGTGTC-3’) were denatured at 95°C for 5 min and chilled immediately on ice for 2 min. Reverse transcription was performed in a 20 μL containing 12 μL RNA, 4 μL of 5 × FS buffer (Invitrogen, USA), 1 μL of dNTP (10 mM each), 1 μL of DTT (0.1 M), 1 μL of specific primer R (5’-CCATTGTAGCTAACATCTGTGTC-3’, 50 μM), 100 U of Superscript™ III; Reverse Transcriptase (Invitrogen, USA), 20 U of RNase Inhibitor (TaKaRa, China). Reaction was carried out at 50°C for 1 h and 70°C for 15 min. 2 μL cDNA was used in conventional PCR and real-time PCR reactions below.

**PCR**

using a pair of primers (F: 5’-GCGAATTCCGGGATCCTTCTGTC-3’, R: 5’-CCATTGTAGCTAACATCTGTGTC-3’) and Ex Taq polymerase (TaKaRa, China), a conventional PCR was carried out with a pre-denaturation at 95°C for 5 min and 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, followed by 72°C for 7 min. PCR product was electrophoresed on 1% agarose gel and stained with ethidium bromide. The correct amplification product showed as a DNA band of about 560 bp.
Real-time PCR

The TaqMan based real-time PCR was performed in a total volume of 25 μL as described in our previous paper [14], and the reaction was performed with a pre-denaturation at 95°C for 5 min, and 40 cycles of denaturation at 95°C for 10 sec and annealing/elongation at 60°C for 40 sec. Fluorescent signal measurements were carried out during the elongation step.

Results

Specificity and Sensitivity of the RT-LAMP

As shown in Figure 2A, RT-LAMP products of RNA from vvIBDV Gx showed a ladder-like pattern on the gel. The reaction also caused change of turbidity, the color inside the tube changed from orange to green that was easily visible to naked eyes under the natural light. The reactions containing samples of common avian pathogens AIV, NDV, IBV, MDV and CAV as well as the negative control showed no product on the gel. Consistently, the color of these negative reactions remained orange.

Upon 10-fold serial dilution, RNA standards with known copy numbers (2.8 × 10^5 copies/μL to 2.8 × 10^0 copies/μL) were used for RT-LAMP. As shown in Figure 2B, RT-LAMP successfully detected as little as 28 copies of RNA molecules, determined by both the agarose gel electrophoresis and color change inspection.

Table 1 Nucleotide sequences of vvIBDVs at the TfiI site

| Strains/isolates | Accession number | Geographic origin | Nucleotides at the restriction site |
|-----------------|------------------|--------------------|-----------------------------------|
| Gx              | AJ444873         | China              | G T T T C                      |
| Harbin-1        | EFS15728         | China              |          |
| SH95            | AY134874         | China              |          |
| GZ/96           | AY598356         | China              |          |
| HK46            | AF092943         | Hongkong           |          |
| Chinju          | AF508176         | Korea              |          |
| SH/92           | AF533670         | Korea              |          |
| OKYM            | DA9706           | Japan              |          |
| SDH1            | AY323952         | Iran               |          |
| BD 3/99         | AF362776         | Bangladesh         |          |
| UPM97/61        | AF247006         | Malaysia           |          |
| UPM94/273       | AF527039         | Malaysia           |          |
| UPM92-04        | AF262030         | Malaysia           |          |
| B00/73          | AYS20909         | Malaysia           |          |
| B00/81          | AYS20910         | Malaysia           |          |
| 94230           | AYS20911         | Malaysia           |          |
| Taskk94         | AF322444         | Indonesia          |          |
| T09             | AY099456         | Nigeria            |          |
| PO7             | AY665672         | Tunisia            |          |
| ks              | DQ292704         | Israel             |          |
| UK661           | AJ318896         | U.K.               |          |
| 02015.1         | AJ879932         | France             |          |
| D6948           | AF240686         | Netherlands        |          |
| Ipumiirim-BR    | AY769978         | Brazil             |          |
| SM-BR           | AY780418         | Brazil             |          |
| MG7             | DQ286035         | Brazil             |          |

Note: The TfiI site was ^GAWTC^W = A or T. Dark dot indicated residue which was identical to the vvIBDV Gx. There was no TfiI site in vvIBDVs.
Evaluation of RT-LAMP for clinical specimens

To evaluate the feasibility of RT-LAMP for detecting IBDV in clinical specimens, 48 clinical specimens were obtained from a wide range of geographic locations and assayed by RT-LAMP. In parallel, conventional RT-PCR and real-time RT-PCR were also performed. As summarized in Table 3, percentage of positive samples detected by conventional RT-PCR, real-time RT-PCR and RT-LAMP were 79.2%, 95.8% and 95.8%, respectively. The results of RT-LAMP and real-time RT-PCR were 100% correlated and the correlation between RT-LAMP and conventional RT-PCR was 83.3%.

The RT-LAMP products of the 46 positive specimens were subsequently digested by Tfi I together with those from the vvIBDV control Gx and non-vvIBDV control D78. Among those, only one clinical specimen showed a 102 bp fragment as well as the negative control from non-vvIBDV.

Table 2 Nucleotide sequences of non-vvIBDVs at the TfiI site

| Strains/isolates | Accession number | Geographic origin | Phenotype | Nucleotides in the restriction site |
|------------------|------------------|-------------------|-----------|-----------------------------------|
| Gt               | DQ403248         | China             | Attenuated| GATTTC<sup>Note</sup>             |
| HZ2              | AF321054         | China             | Attenuated| .                                  |
| JD1              | AF321055         | China             | Attenuated| .                                  |
| CJ801bkf         | AF006694         | China             | Attenuated| .                                  |
| GZ29112          | AF051837         | China             | Attenuated| .                                  |
| NB               | AY319768         | China             | Attenuated| .                                  |
| CEF94            | AF194428         | Netherlands       | Attenuated| .                                  |
| D78              | AF499929         | Luxembourg        | Attenuated| .                                  |
| CT               | AJ310185         | France            | Attenuated| .                                  |
| Cu-1 M           | AF362771         | Germany           | Attenuated| .                                  |
| P2               | X84034           | Germany           | Attenuated| .                                  |
| Edgar T          | AY462026         | USA               | Attenuated| .                                  |
| 002-73           | X03993           | Australia         | Classical | .                                  |
| CU-1             | X16107           | Germany           | Classical | .                                  |
| CS-2-35          | EF418033         | USA               | Classical | .                                  |
| GA-1             | EF418034         | USA               | Classical | .                                  |
| H-30             | EF418035         | USA               | Classical | .                                  |
| P3009            | AF109154         | Taiwan            | Classical | .                                  |
| A-BH83           | DQ187988         | Brazil            | Classical | .                                  |
| STC              | D00499           | USA               | Classical | .                                  |
| Cu1              | D00867           | Germany           | Classical | .                                  |
| FBG-98           | D00868           | U. K.             | Classical | .                                  |
| 52/70            | D00869           | U. K.             | Classical | .                                  |
| IM               | AY029166         | USA               | Classical | .                                  |
| Cu-1 wt          | AF362747         | Germany           | Classical | .                                  |
| Lukert           | AY918948         | USA               | Classical | .                                  |
| Edgar C          | AY918950         | USA               | Classical | .                                  |
| GLS              | AY368653         | USA               | Variant   | .                                  |
| variant E        | AF133904         | USA               | Variant   | .                                  |
| 23/82            | AF362773         | U. K.             | Serotype II; | .                                  |
| OH               | M66722           | Canada            | Serotype II; | .                                  |

<sup>Note</sup> The TfiI site was 5’GAWTCG’ W = A or T. Dark dot indicated residue which was identical to the attenuated Gt. There was a TfiI site in non-vvIBDVs.

Figure 3 Different TfiI restriction patterns of RT-LAMP products of vvIBDV Gx and attenuated Gt strains. RT-LAMP products were digested by TfiI and subjected to a 2% agarose gel. After ethidium bromide staining, DNA band patterns were photographed under a UV transilluminator. Lanes M, DNA marker DL2000 (TaKaRa, China, with 2000, 1000, 750, 500, 250 and 100 bp bands); 1, RT-LAMP product of Gx digested by Tfi I; 2, RT-LAMP product of Gt digested by Tfi I.
non-vvIBDV D78 (not shown), indicating that 97.8% (45/46) of the infected specimens or 93.8% (45/48) of total specimens were infected by vvIBDV.

Discussion

In this study, we developed a RT-LAMP assay for the detection of IBDV and subsequent discrimination of vvIBDV based on its VP5 gene. The use of loop primers in this assay greatly accelerates the reaction [15-20]. We show here that the primers did no cross react with a panel of other common avian pathogens, and the assay had a high sensitivity with the detection limit of 28 copies, which is almost as sensitive as a real-time RT-PCR-based assay for the same virus we developed in the earlier study [14] and 100 times greater than the conventional RT-PCR [17,20-22]. RT-LAMP is more sensitive than the conventional RT-PCR and more convenient than real-time RT-PCR. Another advantage of this assay is that the results can be examined by inspection of color change and examination with agarose gel electrophoresis. Consistency results observed by both methods in this study indicate that a visual inspection is sufficient for a routine test [23]. This is particular useful and can be extremely convenient in a large scale screening process.

In clinical specimens, 93.8% was positive for vvIBDV infection, indicating the severity of vvIBDV infection in many areas of China. Even though vaccination has been widely adopted, vvIBDV can break through high levels of maternal antibodies in commercial flocks [24,25]. Since vaccine was produced by attenuated or classical strains, it is very important and significant that wild isolates of vvIBDV can be distinguished from vaccinated strains.

Sequence analysis showed a SNP in the target sequence of RT-LAMP among IBDV strains. “A” is conserved in classical, attenuated, variant and serotype II strains, creating a Tfi I site in this site, while it is substituted by “T” in typical vvIBDV strains except UK661 that has a “C”. Since this SNP was identified from strains with a wide geographic distribution, so the Tfi I digestion based on this SNP should be reliable and generally work. Although the VP5 of infectious bursal disease virus has been reported to contribute to rival virulence and viral release [26,27], the role of this nucleotide substitution in viral pathogenesis is still unknown. We are yet to determine whether this point mutant may be involved in the virulence or viral release, or it may just be a unique nucleotide tag between vvIBDV and non-vvIBDVs.

Conclusion

In summary, one-step RT-LAMP is a rapid, efficient, sensitive and highly specific assay for the identification of IBDV. In combination with Tfi I restriction analysis, vvIBDV strain can be discriminated from non-vvIBDVs. Owning to these properties, this method showed great promises not only in laboratory test but also in the field and clinical applications.

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Authors’ contributions

YQW and XMW designed this study; YQW wrote the paper; YQW and ZHK carried out this study; YQW, ZHK, HLG and XMW analyzed the data; HLG, YLG, LTQ, HL and XLQ collected the clinical samples; YQW, YLG and FY revised the manuscript critically. All of the authors read and approved the final version of this manuscript.

Competing interests

The authors declare that they have no competing interests.
References

1. Cosgrove AS: An apparently new disease of chicken-avian nephrosis. Avian Dis 1962, 6:383-389.

2. Yuen YM, Xue CY, Wong HC, Qiu L, Chiu TC, Liu YS. Rapid real-time detection of porcine adenovirus by reverse transcription loop-mediated isothermal amplification. J Virol Methods 2009, 157:267-271.

3. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000, 28:e63.

4. Chen HT, Zhang J, Sun DH, Ma LN, Liu XT, Cai XP, Liu YS. Rapid and sensitive diagnosis of foot-and-mouth disease virus by reverse transcription loop-mediated isothermal amplification assay. J Virol Methods 2009, 155:355-60.

5. Feyrefitte GN, Boubis L, Coudiere D, Boulou M, Grandadam M, Tolou HJ, Plumet S. Detection of porcine parvovirus by reverse transcription-loop-mediated isothermal amplification. J Virol Methods 2008, 151:2665-2670.

6. Wang JQ, Xu CY. Rapid detection of porcine reproductive and respiratory syndrome virus by reverse transcription loop-mediated isothermal amplification assay. J Virol Methods 2009, 155:355-60.

7. Xu JT, Zhang ZM, Yin YB, Cui SJ, Xu SZ, Guo YY, Li JD, Wang JL, Liu XC, Han LM. Development of reverse transcription-loop-mediated isothermal amplification for the detection of infectious bursal disease virus. J Virol Methods 2009, 162:267-271.

8. Xue CY, Zhang Y, Zhou QF, Xu C, Li XM, Cao YC. Rapid detection of infectious bursal disease virus by reverse transcription loop-mediated isothermal amplification assay. J Virol Methods 2009, 162:267-271.

9. Wang YM, Zeng XW, Gao HL, Wu CY, Wei P. Changes in VP2 gene during the attenuation of very virulent infectious bursal disease virus strain Gx isolated in China. Avian Dis 2004, 48:77-83.

10. Wang YQ, Qi XL, Gao HL, Gao YL, Lin H, Song XQ, Pei L, Wang XM. Comparative study of the replication of infectious bursal disease virus in DF-1 cell line and chicken embryo fibroblasts evaluated by a new real-time RT-PCR. J Virol Methods 2009, 157:205-210.

11. Blomstrom AL, Halketverydane M, Reid SM, Dukes JP, King DP, Belak S, Berg M. A one-step reverse transcriptase loop-mediated isothermal amplification assay for simple and rapid detection of swine vesicular disease virus. J Virol Methods 2008, 147:188-193.

12. Endo S, Komori T, Ricci G, Sano A, Yokoyama K, Ohori A, Kamei K, Franco M, Miyaji M, Nishimura K. Detection of gp35 of Paracoccidioides brasiliensis by the loop-mediated isothermal amplification (LAMP) method. FEBS Microbiol Lett 2004, 234:99-97.

13. Mao XL, Zhou S, Xu D, Gong J, Cui HC, Qian QW. Rapid detection of foot-and-mouth disease virus by reverse transcription loop-mediated isothermal amplification. J Appl Microbiol 2008, 105:389-397.

14. Nagamine K, Watanabe K, Ohtsuka K, Hase T, Noge N, Totsuka A. Loop-mediated isothermal amplification reaction using a nondenatured template. Clin Chem 2001, 47:1742-1743.

15. Yoneyama T, Kiyohara T, Shimasaki N, Kobayashi G, Ota Y, Notomi T, Totsuka A, Wakiya T. Rapid and real-time detection of hepatitis A virus by reverse transcription loop-mediated isothermal amplification assay. J Virol Methods 2007, 145:162-168.

16. Yang QL, Shi CY, Huang J, Jia KT, Chen XH, Liu H. Rapid diagnosis of tuberculosis in sputa by loop-mediated isothermal amplification method. J Virol Methods 2009, 158:18-23.

17. Cho HS, Park NY. Detection of canine distemper virus in blood samples by reverse transcription loop-mediated isothermal amplification. J Vet Med B Infect Dis Vet Public Health 2005, 52:410-413.

18. Sun ZF, Hu Q, Ren CH, Shen Q. Sensitive and rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHNV) in shrimps by loop-mediated isothermal amplification. J Virol Methods 2006, 131:41-46.

19. Hashimoto Y, Itiba M, Ohta A, Inoue S, Usui C, Asano Y, Yoshikawa T. Discriminating between Varicella-Zoster virus vaccine and wild-type strains by loop-mediated isothermal amplification. J Clin Microbiol 2008, 46:2665-2670.

20. Chettle N, Stuart JC, Wyeth PJ. Outbreak of virulent infectious bursal disease in East Anglia. Vet Rec 1989, 125:271-272.

21. Nuno Y, Otaki Y, Tajima M, Hiraga M, Saito T. Occurrence of acute bursal disease with high mortality in Japan and pathogenicity of field isolates in specific pathogen free chicken. Avian Dis 1992, 36:597-609.

22. Lombardo E, Maravei A, Espina I, Fernandez-Anas A, Rodriguez JF. VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. Virology 2000, 277(2):345-357.

23. Yao K, Goodwin MA, Valkaria VN. Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. J Virol 1998, 72(4):2647-2654.

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