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Reverse Transcription Loop-Mediated Isothermal Amplification for the Rapid Detection of Infectious Bronchitis Virus

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1. Introduction

Infectious bronchitis virus (IBV) is a major cause of disease in domestic fowl and causes an acute, highly contagious disease of the respiratory tracts and sometimes urogential tracts (King and Cavanagh, 1991). Current diagnostic assays for IBV include virus isolation in embryonating eggs, tracheal organ culture, or cell culture immunoassays, and molecular assays that detect the viral RNA (Gelb and Jackwood, 1998). Virus isolation is generally considered the gold standard, however, it is expensive and time consuming because several passages may be required to detect the virus. Immunoassays use IBV-specific monoclonal antibodies to detect the virus in direct or indirect fluorescent antibody and enzyme-linked immunosorbent assay formats. Although more rapid and simpler than virus isolation, immunoassays tend to lack specificity and sensitivity to some extent and can not detect all strains of IBV (Karaca and Naqi, 1993; Karaca et al., 1992; Naqi et al., 1993). Molecular assays for the detection of IBV are used commonly because they provide highly specific and sensitive results and detect viral RNA directly from clinical samples or from virus isolated in a laboratory host system. Although RT-PCR and real-time RT-PCR are the highly sensitive and specific methods (Cavanagh et al., 1992; Jackwood et al., 1997; Keeler et al., 1998; Kingham et al., 2000; Kwon et al., 1993; Zwaagstra et al., 1992; Liu et al., 2003; Callison et al., 2006), the dependence on special equipment limits their extensive use.

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA

and a new stem–loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity. The advantage of LAMP is simple and easy to perform once the appropriate primers are prepared, requiring only four primers, a DNA polymerase and a regular laboratory water bath or heat block for reaction. Another useful feature was reaction products of RT-LAMP could be directly observed by the addition of dyes. By combination with reverse transcription, LAMP can also amplify RNA sequences with high efficiency (Notomi et al., 2000).

Reverse transcription LAMP (RT-LAMP) has been applied successfully for the detection of influenza A virus, severe acute respiratory syndrome coronavirus and Newcastle disease virus (Chen et al., 2008; Hong et al., 2004; Pham et al., 2005; Poon et al., 2005). In the present study, the reference strain IBV01, IBV02 and IBV03 were from Chinese Veterinary Microorganism Conservation Center (CVMCC), the strains of IBV01 and IBV02 were Massachusetts serotype and IBV03 was the T strain. IBV01 was used to standardize the IBV RT-LAMP assay. The vaccine strains of H120 and M41 were supplied by a local vaccine manufacturer. The othe vriruses, including Newcastle disease virus (NDV), avian reovirus (ARV), and infectious laryngotrachietis virus (ILTV) were also from CVMCC and used to examine the specificity of IBV RT-LAMP. RNA transcripts corresponding to the nucleocapsid (N) phosphoprotein gene of IBV01 genome were generated to use as standards in the sensitivity analysis of the assay. A series of the 10 times dilutions spanning from 1 to 10^5 copies/tube was used as template. Briefly, RNA was extracted from IBV strains using the RNeasy Mini Kit (Qiagen). The purified RNA was resuspended in diethylpyrocarbonate treated water and used in the RT-PCR reaction. The amplified product of N gene was cloned into the pCR-XL-TOPO vector (Invitrogen Inc., Shanghai, China) according to the manufacturer's directions and sequenced to verify its accuracy. The recombinant plasmid pCR-N was linearized and gel purified and used as template with a RiboMax T7 In Vitro Transcription System (Promega, Madison, WI) according to the manufacturer's recommendations. The length of the RNA transcripts was verified by agarose gel analysis, and the RNA of N gene was quantitated using UV spectrophotometry at 260 nm.

Four primers of FIP, BIP, F, and B for the RT-LAMP test were designed by targeting the conserved regions of N gene and listed in Table 2. RT-LAMP was performed in 25 μL of a mixture containing 2 μL of the genomic RNA, 40 pmol (each) of primers FIP and BIP, 5 pmol (each) of primers F and B, 1 U of the THERMO-X reverse transcriptase (Invitrogen) and 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA) with the corresponding buffer, respectively. Amplification was carried out at 64^0C for 15, 30, 45, 60, 75min, respectively, and then terminated by incubation at 80^0C for 2 min. The electrophoresis analysis indicated that 45 minutes are enough for IBV RT-LAMP in the study. The products of the reaction were also inspected by the naked eye following the addition of 1μl of SYBR Green I dye to the tube. All the strains tested by RT-LAMP were also identified by RT-PCR and sequenced. Each assay was conducted in triplicates. The details of primers (NF and NR) and condition for RT-PCR assay for the detection of IBV have been previously described (Zwaagstra, et al. 1992), with minor modifications (Table

1). A perfect correlation was found for all of the IBV strains which were positive by the RT-LAMP and RT-PCR and no cross reaction of IBV RT-LAMP was tested with NDV, ARV and ILTV (Table 2).

Method	Primer	Position [a]	Sequence
RT-LAMP	F	834-853	5'-CGTACTAAAGGTAAGGAGGG-3'
	B	1247-1228	5'-CTCCTCATCTGAGGTCAATG-3'
	FIP	1061-1041	5'-ACAAATTTTTACATAATTATCA +TTTT+
		877-896	ATGAGGAGGGTATTAAGGAT-3'
	BIP	1136-1157	5'-ACCTGCAACAAGAGGAAATTCT +TTTT+
		1202-1183	CTTTGGCTTTTTCTCCTTCT-3'
RT-PCR[b]	NF	24-40	5'-GTCTTGTCCCGCGTGTA-3'
	NR	461-445	5'-ACCCTTACCAGCAACCC-3'

[a] Position is marked according to the sequence of IBV strain (GenBank accession number EU889030)
[b] The primers of NF and NR were designed according to the method described previously by Zwaagstra, et al. (1992), with minor modifications.

Table 1. RT-LAMP and RT-PCR primers designed for detection of IBV.

Strain	Result by	
	RT-LAMP	RT-PCR
IBV01	+	+
IBV02	+	+
IBV03	+	+
H120	+	+
M41	+	+
NDV	–	–
ARV	–	–
ILTV	–	–

Table 2. The specificity of the IBV RT-LAMP assay compared with RT-PCR

For further evaluation of RT-LAMP assay with clinical specimens, 187 specimens of blood, kidney and lung tissue were obtained from IBV-infected chicken. The result of RT-LAMP was analyzed and compared with RT-PCR with all clinical specimens. The general detection rates of IBV RT-LAMP and RT-PCR for above mentioned different clinical samples were 99.5% and 98.4%, respectively. In general, both assays showed higher sensitivity for blood and lung samples than kidney (Table 3). The results indicated that this diagnostic technique was reliable for the detection of IBV in blood, kidney and lung tissue samples. Blood seems the preferred samples during the early stage of infection, which may have a higher predictive value of monitoring an outbreak.

Specimen type	No. of positive samples	No. (%) of samples with indicated result by:	
		RT-PCR	RT-LAMP
Blood	88	88 (100)	88 (100)
Kidney	62	60 (96.8)	61 (98.3)
Lung	37	36 (97.3)	37 (100)
Total	187	184 (98.4)	186 (99.5)

Table 3. Comparative evaluation of RT-LAMP assay with RT-PCR for 187 clinical samples

The result indicated detection limit of IBV RT-LAMP was 10 copies/tube (Fig. 1). In addition, the reaction time of RT-LAMP method is 45 min, which is more rapid than conventional RT-PCR, and the reaction only needs a laboratory water bath. Another useful feature was RT-LAMP products could be directly observed by the addition of dyes.

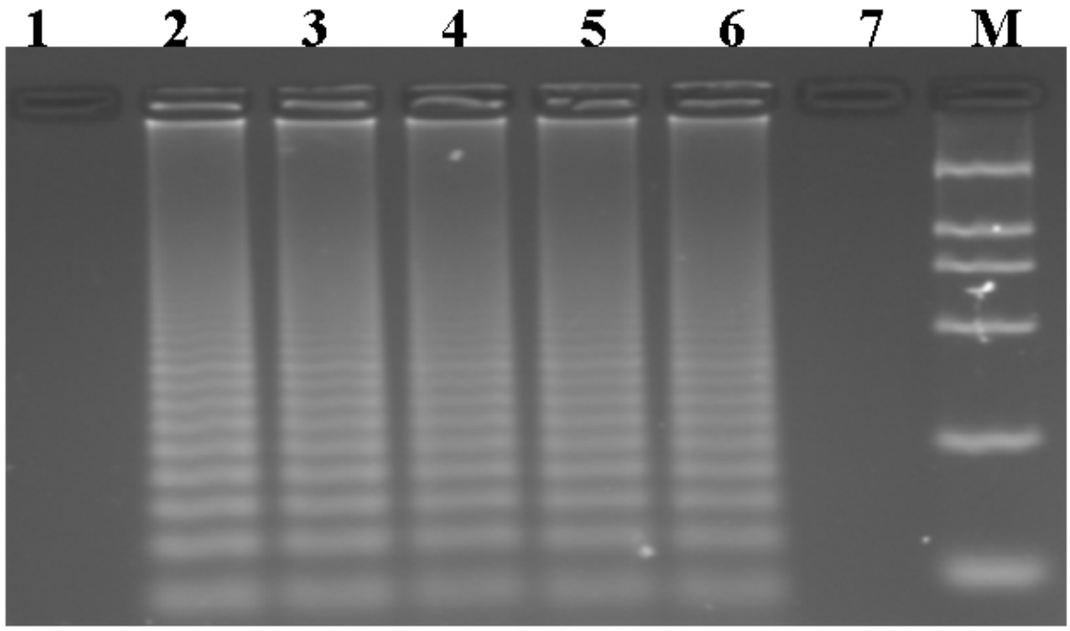

Fig. 1. Sensitivity of RT-LAMP determined by agarose gel electrophoresis of RT-LAMP products from spiked with 10-fold serial dilution of IBV RNA. Lines M, DNA marker DL2000; Lanes 1 to 6, different IBV RNA copy numbers of RT-LAMP
(1, 10, 10^2, 10^3, 10^4 and 10^5copies/tube, respectively); lane 7, negative control.

From a practical point of view, RT-LAMP is more suitable as a routine diagnostic tool, especially in clinics in which complicated equipment such as thermal cycling machines and electrophoresis apparatus are not available. In addition, RT-LAMP has a potential for field diagnosis. Nonetheless, the reliability of this assay should be further evaluated by large-scale investigation.

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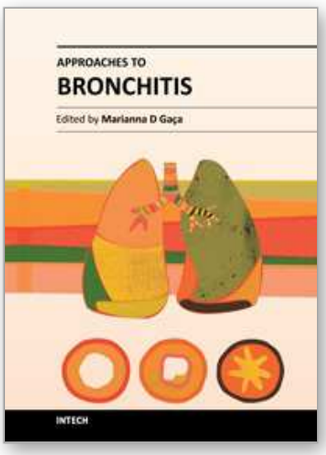

Approaches to Bronchitis

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The aim of this book is to present some recent and interesting findings in the field of bronchitis, which will serve as a supplement to the book Bronchitis. In particular, this volume focuses on the successful use and development of novel tools in the diagnostics and treatment of bronchitis. Contributions include clinical case studies, the impact of air pollution on bronchitis, the presentation and diagnosis of the respiratory disease eosinophilic bronchiolitis, primary ciliary dyskinesia, the development of a method for the swift detection of the infectious bronchitis virus and studies investigating the successful use of alternative medicines in the treatment of bronchitis. The editor would like to thank the authors of the chapters who have contributed to this book and hopes that this will book not only supplement the book on Bronchitis, but may increase interest in the subject.

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