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Rapid Detection of H4 Subtype Avian Influenza Viruses by Reverse Transcription Loop-Mediated Isothermal Amplification Assay

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Abstract: Avian Influenza Viruses (AIV) have been isolated from a wide range of avian species through out the world. And the H4 subtype AIV is one of the most predominant subtypes among Low Pathogenic AIVs (LPAIVs). Recently some H4 subtype AIVs can be found and isolated in South China from chicken or duck and have become the main popular LPAIVs subtype. Consequently, the development of a rapid, simple, Sensitive Detection Method for H4 subtype AIVs is required for us. A Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) assay was developed to detect the H4 subtype AIVs visually. Specific primer sets targeting the sequences of the Hemagglutinin (*HA*) gene of H4 subtype AIVs were designedand RT-LAMP assay reaction conditions were optimized, ordinary RT-PCR and virus isolation assays were compared. The established LAMP assay was performed for 50 min in a water bathand the amplifications were visualized directly Under ultraviolet (UV) light. The detection limit of the RT-LAMP assay was 10 EID 50 mL^{-1} RNA of virus which was more sensitive than that of ordinary one-step RT-PCR (100 EID50 mL^{-1} RNA of virus). The specificity results indicated that the assay had no cross-reactions with other subtype AIVs. Furthermore, the result of clinical samples by the H4-subtype-specific RT-LAMP were consistent with that of virus isolation which was superior to ordinary RT-PCR. In this study, the established RT-LAMP assay with high sensitivity was performed completely within only 50 minand the amplification results were visualized by adding fluorescence reagent. The newly developed simple assay can be used as one important method for detecting H4 subtype AIVs in clinical conditions with no need for specialized equipment and high techinique.

Key words: Loop-mediated isothermal amplification, H4 subtype, avian influenza, assay, gene

INTRODUCTION

Avian Influenza (AI) is an infectious disease caused by influenza A viruses that mainly attacks poultry that affects the systemic or respiratory organs of chickens, turkeys, ducksand geese (Kageyama *et al*., 2013; Zhao *et al*., 2012; Morens *et al*., 2012). The classification of the Avian Influenza Virus (AIV) strains is based on the Hemagglutinin (*HA*) gene and Neuraminidase (*NA*) gene (Tsukamoto *et al*., 2012). Results from surface antigen identification show that the influenza virus extracted from poultry have 16 types of HA and 9 types of NA which can be combined to generate numerous subtypes. Some studies showed that Low-Pathogenic Avian Influenza Viruses (LPAIVs) can provide the newly gene for the infected AIV by gene rearrangement. The H4 subtype AIV belongs to the LPAIV and H4 subtype strains has been prevalent in China in recent years (Ninomiya *et al*., 2002; Lin *et al*., 1994). To date this subtype is also reported in South Korea, Russiaand other countries (Veits *et al*., 2012; Kang *et al*., 2013; Beare and Webster, 1991). In 1999, one strain of H4N6 virus was extracted from a pig in Canada. Sequence analysis showed that the 226L of the *HA* gene enabled the virus to infect the pig. This phenomenon shows that the H4 subtype AIV is gradually adapting to the body of the mammal (Karasin *et al*., 2000).

Loop-Mediated Isothermal Amplification (LAMP) is a new technology of the isothermal nucleic acid amplification which amplifies using a BstDNA polymerase that has a strand displacement activity at 65°C (Le Roux *et al*., 2009; Kiatpathomchai *et al*., 2007). A visual study by white turbid precipitation was conducted to determine whether the positive reaction product can generate white turbid precipitation. Green fluorescence can be found by adding SYBR Green I dye into the product and then observed the product under ultraviolet rays. The reaction can be completed within 1 h in a water bath. This technology has good specificity and simple operation. It is also rapid and very fit for application in the basic level and clinical site. LAMP is currently applied to

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detect various primary animal pathogens such as the H5, H3 and H9 subtypes AIV, Infectious Bronchitis Virus (IBV) and Newcastle Disease Virus (NDV) (Chen *et al*., 2008; Xu *et al*., 2009; Imai *et al*., 2006; Pham *et al*., 2005). In this research, researchers established the RT-LAMP rapid detection assay for H4 subtype AIV.

MATERIALS AND METHODS

Strains: Experiments involving the extraction and identification of H4 (JN strain) and H9 (CL strain) subtypes AIVs were performed in the Laboratory of Poultry Institute, Shandong Academy of Agricultural Sciences. The H1 (XM strain) and H5 (WF strain) subtypes AIVs were extracted, identified and preserved by Key Laboratory Research Office of the Prevention and the Control of the Animal Origin Amphixenosis of South China Agricultural University.

RNA extraction: The genomic viral RNA was extracted from the cultures obtained from 9-11 day SPF chicken embryo by using the MiniBEST viral RNA Extraction kit (TaKaRa, Japan) according to the manufacturer's protocol. The RNA was eluted in a final volume of 50 μL of elution buffer and stored at -80°C until used.

Design of primers for the RT-PCR: One-step RT-PCR was performed with AIVs (H1, H4, H5 and H9 subtype) specific primers designed from the *hemagglutinin* genes. These specific primers for one-step RT-PCR used in this study were listed in Table 1.

Design of primers for the RT-LAMP: According to the *HA* gene sequences of the H4 subtype AIV in GenBank (Accession No. JX454706) and the sequences of the JN isolate sequenced by us the primer sets of RT-LAMP assays were designed using the LAMP primer design software Primer Explorer V4. The LAMP primer set comprising two outer primers (Forward primer F3 and Backward primer B3), two inner primers (Forward inner primer FIP and Backward Inner Primer BIP). The details for the primers were shown in Table 2.

RT-LAMP: The RT-LAMP reaction was carried out in a total 25 μL reaction volume containing 40 pmol each of the primers FIP and BIP, 10 pmol each of the outer primers F3 and B3, 10 mM deoxynucleoside triphosphate (Takara), 0.8 M betaine (Ferments), 8 U of Bst DNA polymerase (NEB), 10 U of the Avian Myeloblastosis Virus (AMV) reverse transcriptase (TaKaRa) and 2 μL of viral RNA. The amplification reaction was performed in different temperature of 58, 60, 62, 64 and 66°C for 40, 50 and 60 min, respectively to find the optimal reaction temperature and time and followed by heating at 85°C for 2 min to terminate the amplification reaction. Negative and positive controls were carried out in separate runand all precautions to prevent cross-contamination should be accepted.

RT-PCR: The amplification was carried out in a 50 μL total reaction volume by using the TaKaRa One-Step RT-PCR kit with 50 pmol of forward and reverse primers and 2 μL of RNA according to the manufacturer's protocol. The reaction condition of RT-PCR was optimized to get the highest sensitivity of amplification. Finally, the thermal profile of RT-PCR for was 50°C for 50 min and 94°C for 3 min followed by 35 cycles of 94°C for 90 sec, 52°C for 90 sec, 72°C for 90 sec and a final extension cycle of 72°C for 10 min.

Sensitivity and specificity of RT-LAMP and RT-PCR: The specificity of RT-LAMP reaction was carried out for

Table 1: Sequences of primers designed for RT-PCR assay

Subtype	Length (bp)	Genbank No.	Primers (5'-3')
H1	1608	JX080642	Forward-TTATCATGCGAACAATTCAACAGAC Reverse-GACCCATTAGAGCACATCCAGAA
H4	1529	JX454706	Forward-TGTTTCTGCTCGTAGC Reverse-AATTTGGAATCGGTTGT
H5	589	CY146708	Forward-CATTCCACAACATACACCCTC Reverse-GTTCCGTTTTTTACACTTCC
H9	404	JF795136	Forward-GCCTGCTAGATCAAGTAGAGG Reverse-TGGAACCCAATGCCCTCTTC

Table 2: Sequences of primers designed for RT-LAMP assay

Primers	Position	Sequence
F3	1356-1375	TGTAACTGACTCGGAGATGA
B3	1543-1524	GGAATCGATTGTTGATTGCT
FIP (F1c+TTTT+F2)	1438-1417+TTTT+1377-1398	ACCCATTTCCTTTGTCTTCAGCTTTTCA AGCTCTTTGAAAGAGTAAGG
BIP (B1c+TTTT+B2)	1439-1463+TTTT+1515-1493	GCTTCGAAATATTCCACAAATGTGATTTTT ATAGACATCATGATCATAGGTCC

strain JN (H4 subtype), HPAIV strain WF H5 subtype, LPAIV strain CL (H9 subtype) and XM (H1 subtype) as contrastive specimens. The sensitivity of the RT-LAMP assay for the detection of H4 subtype was determined by testing serial 10 fold dilutions of virus that had previously been quantified by EID 50 mL^{-1} assay and compared with that of conventional RT-PCR. Following incubation for RT-LAMP or RT-PCR, a 10 μL RT-LAMP or RT-PCR products were electrophoresed with a 1% agarose gel in TAE buffer followed by staining with Ethidium Bromide (EB) and visualization on an UV transilluminator. The products were purified using MiniBEST Agarose Gel DNA Extraction Kit and were sequenced by TaKaRa Company.

RT-LAMP visualization: The amplification results were analyzed using agarose gel electrophoresis under UV light. Meanwhile, in order to facilitate the field application of the RT-LAMP assay, the monitoring of RT-LAMP amplification was also carried out with a naked-eye inspection for white magnesium pyrophosphate precipitations in the tube and under UV light following the addition of 1 μL of SYBR green I (1:1000) dye to the tube.

RT-LAMP assay with attacked-virus samples: Selected 40, 14 day old ducks were randomly divided into two groups: the first group of 20 as drug group for the viral attacking, attacked agents volume was 10^5 EID_{50} mL^{-1} by calculation using the Reed-Muench Cumulative Method. The second group as the control group, attacked the same dose of saline injection. Two groups were recorded daily mortality for 10 days and collected cloacal swab samples for two times. After 10 days, no death or disease were found in attacking ducks and the control ducks. The 40 random cloacal swab samples for the attacking group and 20 cloacal swab samples for the control group were made up of 0.05 M Phosphate Buffered Saline (PBS) containing penicillin (10000 units mL^{-1}), streptomycin (10 mg mL^{-1}), processed as samples for RT-LAMP, RT-PCR and virus isolation, respectively.

RESULTS

The specificity of RT-LAMP assay for detection of H4 subtype AIVs: Through multiple optimization of RT-LAMP assay for detection of H4 subtype AIVs, the RT-LAMP reaction was optimized in a 25 μL total reaction mixture and performed in a conventional water bath for 50 min at 63°C and then for 5 min at 80°C to terminate the reaction. The positive results of RT-LAMP assay showed typical ladder pattern by 1.0% agarosegel electrophoresis (Fig. 1). In this figure, subtypes of H1, H5 and H9 samples showed no typical ladder pattern but their one-Step RT-PCR confirmed the presence of corresponding viral RNA, respectively (Fig. 1). The H4 positive samples showed a strong green fluorescence under UV light (Fig. 2). The products of the RT-LAMP assay were confirmed as H4 subtype AIV by sequencing analysis. So, the results of the H1, H5 and H9 samples showed no typical ladder pattern by 1.0% agarose gel electrophoresis and also had no color change under daylight or UV light which indicated that the RT-LAMP assay for H4 subtype had no cross reaction with other subtype AIVs.

The sensitivity of RT-LAMP assay for detection of H4 subtype AIVs: The sensitivity of the RT-LAMP assay for the detection of H4 subtype was determined by testing

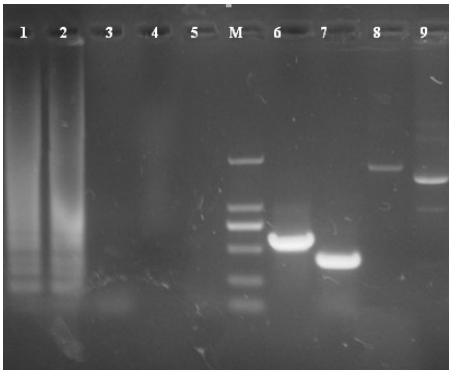

Fig. 1: Amplified products of the RT-LAMP and RT-PCR assay were observed on a 1% agarose gel electrophoresis under UV. Two H4 subtype positive amplifications showed a ladder-like pattern (lane 1, 2) but lane 3, 4 and 5 (H5, H9 and H1 subtype) were negative. Amplified products of RT-PCR of H5, H9, H1 and H4 subtype were observed in lane 6~8, respectively; M: Marker DL 2000; 1, 2: JN isolate (H4 subtype); 3: WF isolate (H5 subtype); 4: CL isolate (H9 subtype); 5: XM isolate (H1 subtype); 6: WF isolate (H5 subtype); 7: CL isolate (H9 subtype); 8: XM isolate (H1 subtype); 9: JN isolate (H4 subtype)

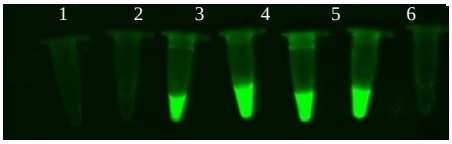

Fig. 2: The inspection of the RT-LAMP reaction stained with SYBR GreenI under UV light. Tubes 3~6 H4 subtype isolate showed green color; tubes 1, 2, 7 H5, H9 and H1 had no green color

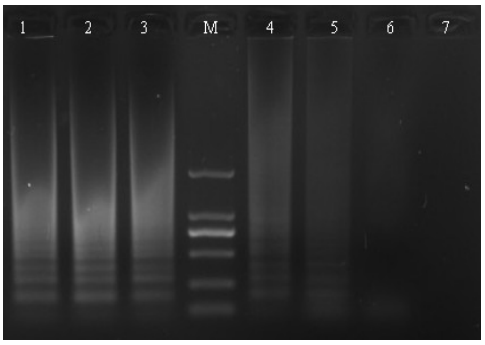

Fig. 3: Amplified products of the RT-LAMP assay are observed on a 1% agarose gel electrophoresis under UV in the sensitivity of the RT-LAMP assay; M: Marker DL2000; 1-7: Amplified products of serial 10 fold dilutions of virus in RT-LAMP, respectively

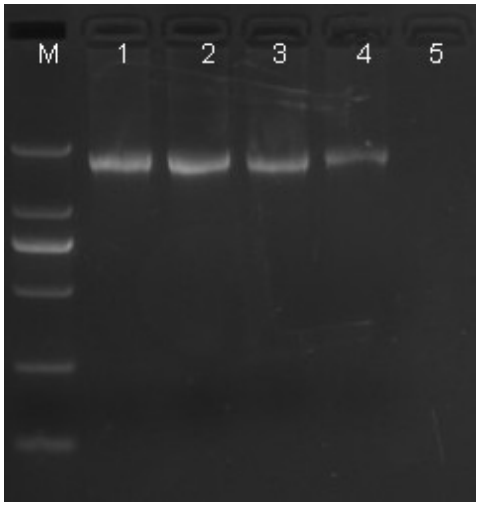

Fig. 4: Amplified products of the RT-PCR assay are observed on a 1% agarose gel electrophoresis under UV in the sensitivity of the RT-PCR assay. M: Marker DL2000; 1-5: Amplified products of serial 10 fold dilutions of virus in RT-LAMP, respectively

serial 10 fold dilutions of virus. The detection limit of RT-LAMP assay was 10EID50 mL^{-1} total RNA of virus which was 10 fold higher than that of RT-PCR (100EID50 mL). So, RT-LAMP assay was more sensitive than RT-PCR. The results of the visual detection of sensitivity tests were shown in Fig. 3 and 4.

Evaluation of RT-LAMP assay with clinical samples: A total of 40 random cloacal swab samples for attacking and 20 contrast samples were collected from duck and were

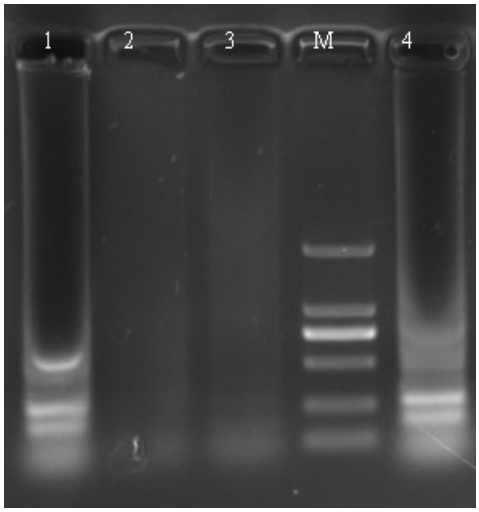

Fig. 5: Amplified products for RT-LAMP assay with some attacked-virus samples; M: Marker DL2000; 1, 4: positive attacked-virus samples; 2, 3: the control healthy ducks

tested by RT-LAMP, RT-PCR and virus isolation, respectively. The results of virus isolation showed that there were 37 positive samples of H4 subtype AIVs among all cloacal swab samples which was consistent with that of RT-LAMP assay. However, only there were 34 positive samples by RT-PCR. Part of amplified products for RT-LAMP assay with some attacked-virus samples were showed in Fig. 5.

DISCUSSION

H4 subtype AIV is one low-pathogenic AI. However, this subtype can spread among poultry animals causing invisible infection in the poultry. It can also cause antigenic drift or change to form a new strain with stronger toxicity, thus, early screening for this antigen are very important. The general diagnostic methods for the AIV include virus isolation, Hemagglutination (HA) Hemagglutination Inhibition (HI) test, Enzyme-Linked Immunosorbent Assay (ELISA), immunohistochemistry and immunofluorescence techniques (Yuen *et al*., 1998; Spackman *et al*., 2002). These methods have their respective advantages and disadvantages such as long testing period, low sensitivity, weak specificity and unsuitability for detection in the basic-level live poultry market or the large-scale import and export samples. Therefore, the establishment of a simple, Rapid and Accurate Detection Method that can be applied in the basic level and on-site is necessary. The Loop-Mediated Isothermal Amplification (LAMP) assay is a Nucleic Acid

Amplification Method developed by Notomi *et al.* (2000) that does not require any specialized equipment.

The specificity and affinity of the HA protein for AIV to the receptor is one of the key factors in determining the host tropism and transmission capacity. Based on HA gene this study establishes an isothermal nucleic acid amplification technology to detect the H4 subtype AIV. The result showed that the RT-LAMP Method established in this study has excel specificity and high sensitivity for the detection of the H4 subtype AIV. The minimum RNA detection value of this method is 10EID50 mL which is ten times that of the ordinary RT-PCR. The negative aspects of the LAMP Method is when the reaction liquid tube cap is opened, the reaction product easily forms aerosol that pollutes the environment, reagentand experimental equipment so causes some false positive results. Therefore, throughout the whole experiment using LAMP technology, the protective operation should be carefully implemented (Peng *et al.*, 2011; Nagamine *et al.*, 2001; Kaneko *et al.*, 2007).

CONCLUSION

This study establishes the LAMP method for H4 subtype AIVs that is simple, fast and cheap. It also has good specificity and high sensitivity which makes it suitable for the H4 subtype AIVs detection and surveillance at the border ports and on-site at the basic levels. So this method is adaptive in the prevention and control the H4 AIV disease.

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