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NOTE *Virology*

Development of Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP) Assay for Detection of Avian Influenza Viruses in Field Specimens

Sakar SHIVAKOTI[1)], Hiroshi ITO[1,2)], Toshiyuki MURASE[2,3)], Etsuro ONO[2,4)], Hiroki TAKAKUWA[5)], Tetsu YAMASHIRO[6)], Koichi OTSUKI[2,5)] and Toshihiro ITO[1,2)]*

[1)]Laboratory of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680–8553, [2)]Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori 680–8553, [3)]Laboratory of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori 680–8553, [4)]Laboratory of Biomedicine, Center of Biomedical Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812–8581, [5)]Avian Influenza Research Center, Kyoto Sangyo University, Kyoto 603–8555 and [6)]Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852–8523, Japan

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ABSTRACT. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is an established gene amplification method for rapid diagnosis of various infectious diseases. In order to detect avian influenza viruses, particularly in field specimens, specific primers targeting the matrix gene were designed. Thirty-four virus samples, including isolates from wild and domestic avian hosts belonging to various geographical areas, were used to confirm the validity of the primers. All samples were confirmed to be positive in less than 1 hr. The RT-LAMP assay was also able to detect avian influenza virus in the various field samples, such as swabs, tissues, and feces. These results indicate that the developed RT-LAMP assay with uniquely designed primers is potentially useful in comprehensive avian influenza surveillance.

KEY WORDS: avian influenza virus, LAMP primers, Matrix gene, rapid detection, RT-LAMP.

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Influenza A viruses belong to the family Orthomyxoviridae, having an eight-segmented, single-stranded, negative-sense RNA genome [23], and are further classified into 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes [6]. Influenza A viruses have been isolated from a number of different animal hosts, including humans, pigs, horses, seals and whales, as well as a variety of domestic and wild birds [21, 22]. To date, most HA/NA combinations have been identified in the domestic and wild bird reservoir [23]. Surveillance of influenza A virus infection and outbreaks in non-primate mammals and birds is essential in understanding the ecology of influenza viruses, and to determine the molecular basis of host range transmission and spread in new hosts.

Various molecular diagnostic tests have been used for detection of influenza A viruses, such as reverse transcription-PCR (RT-PCR) [5], microarray [4], nucleic acid sequence-based amplification (NASBA) [3], real-time RT-PCR [16], mismatch amplification mutation assay (MAMA) PCR [8] and multiplex RT-PCR assay [2, 24]. However, most of these methods require expensive and specialized instruments and reagents, and therefore may not be readily applicable, particularly in diagnostic laboratories in developing and underdeveloped countries or in frontline laboratories. In contrast to other molecular techniques, loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method in which reagents react under isothermal conditions with high specificity, efficiency and rapidity [14].

The method is applicable to amplification of RNA templates by combination with a reverse transcription reaction (RT-LAMP), and this approach has been applied successfully to the detection of numerous viruses, such as West Nile virus [15], viral hemorrhagic septicemia virus (VHS) [19], Spring viremia of carp virus [18], respiratory syncytial virus (RSV) [20], H3 swine influenza virus [7], infectious bursal diseases virus [25], hepatitis E virus [11] and Newcastle disease virus [12]. Moreover, RT-LAMP has been used for diagnosis of infection by influenza viruses, including H5 and H9 subtypes [1, 10]. However, this method has not been reported for the detection of all subtypes of influenza A virus. In this study, we have selected the most conserved region of the matrix gene sequence of the influenza A virus in order to design common primers and to sensitively and specifically detect a wide range of avian influenza viruses using the RT-LAMP assay.

Thirty-four avian influenza virus isolates and three non-avian influenza viruses were used in order to determine the sensitivity and specificity of the present RT-LAMP assay. These strains represent viruses from wild and domestic avian hosts from various geographical distributions in both Eurasian and North American regions over the 60-year period prior to 2009.

In addition to infected allantoic fluid, tracheal swabs from a whooper swan infected with HPAI virus A/whooper swan/Aomori/1/2008 (H5N1), fecal samples from a whistling swan and duck infected with A/whooper swan/Shimane/148/09 and A/duck/Saga/62/08, respectively, and tissue and swab samples from the carcass of a mountain hawk-eagle, collected from Kumamoto Prefecture, infected with HPAI

* CORRESPONDENCE TO: ITO, T., Department of Veterinary Public Health, Faculty of Agriculture, Tottori University, 4–101 Minami, Koyama-cho, Tottori 680–8553, Japan.
e-mail: toshiito@muses.tottori-u.ac.jp

virus A/mountain hawk-eagle/Kumamoto/01/07 (H5N1) were used to determine the sensitivity of the present RT-LAMP assay for field specimens. RNA isolation of the H5N1 HPAI virus samples was performed in a Biosafety level 3 laboratory at Tottori University.

Genomic viral RNA was extracted from 140 μl of allantoic fluid or 10% suspensions of swab, tissues and fecal samples using the QIA amp viral RNA mini kit (Qiagen, Valencia, CA, U.S.A.) in accordance with the manufacturer's protocols. RNA was eluted from QIAamp Mini spin columns in a final volume of 60 μl of elution buffer. The eluted RNA concentration was determined with a ND-1000 spectrophotometer (Nano Drop, ND-1000, NanoDrop Technologies, Wilmington, DE, U.S.A.) and the RNA concentration of each sample was adjusted to 60–80 $ng/\mu l$. The RNA samples were stored at –80°C until use.

The matrix gene sequences of Influenza A viruses from wild and domestic avian hosts belonging to different geographical distributions were obtained from the influenza A database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). As identifying conserved regions for primer construction was nearly impossible with a single alignment from all the available viral sequences, total virus sequences were divided into the following bird groups: Eurasian wild; Eurasian domestic; North American wild; and North American domestic. All sequences were aligned (BioEdit version 7.0.8.0) and conserved regions for each group were traced out. One set of primers from each group, with each set having four specific primers, including two inner primers (one forward inner primer, one backward inner primer) and two outer primers (forward outer primer and backward outer primer), recognizing six distinct genomic sequences of the matrix gene of influenza A virus, and all falling in conserved regions, were designed manually following the standard parameters of the prime explorer LAMP primer designing software (http://primerexplorer.jp/e/).

RT-LAMP was carried out using a Loopamp RNA Amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). Briefly, 5 μl of extracted RNA was added to 20 μl of master mix containing 12.5 μl of 2× reaction mixture, 1 μl of enzyme (mixture of Bst polymerase and AMV reverse transcriptase), 40 pmol of each inner primer and 5 pmol of each outer primer, and distilled water to make a final volume of 20 μl. The mixture was incubated using a Loopamp real-time turbidimeter (RT-160C; Eiken Chemical). Turbidity of the reaction was measured by real time and the result was indicated with the graph in the monitor of real time turbidimeter verifying the startup of the amplification. RNA was extracted from the allantoic fluid samples of 37 strains and RT-LAMP was carried out separately with all the four sets of primers for 120 min at 63°C constant temperature. After determining the best set of primers in terms of sensitivity, specificity and speed, RT-LAMP was further carried out at 60 to 65°C in order to determine the optimum temperature. The optimum temperature was recorded as 63°C and time was set for 60 min, followed by 5 min at 80°C in order to terminate the reaction. The experiment was replicated twice and average detection time for each sample was calculated.

For the visual detection of RT-LAMP results, fluorescent detection reagent (Eiken Chemical) was added to the master mix (1 μl/test), followed by incubation for 60 min in a conventional water bath maintained at 63°C, followed by 5 min at 80°C. The same RNA extracts from six different avian influenza A viruses (A/duck/Memphis/546/74, A/Turkey/Ontario/6118/68, A/mountain hawk-eagle/Kumamoto/01/07, A/chicken/Germany/N/49, A/whooper swan/Aomori/ 2/2008 and A/chicken/VN/G14-TC5&6/08) and one influenza B virus, B/Lee, used for the RT-LAMP assay, were used for this purpose and the products were observed under a UV lamp (λ= 365 nm) after termination of the reaction.

In order to design specific primers for detecting a wide range of avian influenza viruses, a total of 823 sequences were classified into Eurasian wild, Eurasian domestic, North American wild and North American domestic bird groups, and were then aligned. One set of primers from each group was designed, based as far as possible on conserved regions. Of the four sets of primers, the set with the best results (prepared from the North American wild group) was selected. The primers used in this study are listed in Table 1. All avian influenza viruses were detected in less than 60 min and as rapidly as 27 min (Table 2). The three non-avian influenza viruses (infectious bronchitis virus, influenza B virus and Newcastle disease virus) were not detected. These results indicate that there is no cross-reactivity among non-avian influenza viruses, and that the primers are specific to the avian influenza virus (Table 2).

The detection limit of the RT-LAMP assay in the 60-min amplification period was assessed by titrating and serially diluting (10-fold) A/chicken/Ibaraki/01/05 (Ibaraki/05). For viral titration, 100 μl of 10-fold diluted Ibaraki/05 virus was inoculated into five SPF embryonated hen's eggs and the median embryo infectious dose (EID_{50}) was calculated according to the Reed and Muench formula [17]. From each dilution, RNA was extracted and RT-LAMP was carried out. The results showed that the detection limit of RT-LAMP within 60 min was a 10^5 fold dilution of the initial virus titer (i.e., approximately 10^3 EID_{50}/reaction tube), as determined by electrophoresis with LAMP-specific ladder-shaped bands (Fig. 1).

The ability of the developed RT-LAMP analysis to detect avian influenza viruses in swab, tissue and fecal samples was assessed by testing three tissue samples and two swab samples of a H5N1 HPAI virus A/mountain hawk-eagle/Kumamoto/01/2007-infected mountain hawk-eagle and a tracheal sample from a whooper swan infected with the H5N1 HPAI virus A/whooper swan/Aomori/1/2008. RT-LAMP confirmed all the swab and tissue samples to be positive when the reaction was carried out for 60 min (Table 3). Viral RNA extracted from frozen fecal samples of wild waterfowl infected with A/whistling swan/Shimane/148/2009 and A/duck/Saga/62/2008 were also confirmed to be positive by RT-LAMP assay. Unlike A/duck/Saga/62/2008, the A/whistling swan/Shimane/148/2009-infected fecal sample was confirmed to be positive in 67 min when the

Table 1. Oligonucleotide primers used for RT-LAMP amplification of matrix gene of avian influenza viruses

Primer name	Type	Length(s)	Genome position	Sequence (5'–3')
F3	Forward outer primer	19-mer	26–44	ATGAGTCTTCTAACCGAGG
B3	Reverse outer primer	19-mer	238–256	TCTACGCTGCAGTCCTCGC
FIP	Forward inner primer	37-mer; F1C,18-mer F2, 19-mer	F1c;94–111 F2;45–63	TCAAGTCTCTGCGCGATC-TCGAAACGTACGTTCTCTC
BIP	Reverse inner primer	39-mer; B1C,20-mer B2,37-mer	B1C;167–186 B2;219–237	ACAAGACCAATCCTGTCACC-TCACTGGGCACGGTGAGCG

Table 2. Detection of avian influenza viruses of different subtypes by RT-LAMP assay

Virus	Detection time in min
Eurasian isolate	
A/duck/Hong Kong/319/78 (H2N2)	30
A/duck//Hokkaido/8/80 (H3N8)	35
A/duck/Vietnam/G114C20/2006 (H3N8)	35
A/duck//Ukraine/1/63 (H3N8)	34
A/chicken/Vietnam/G14-TC5&6/2008 (H3N8)	35
A/whistling swan/Shimane/227/2001 (H3N9)	28
A/budgregriar/Hokkaido/1/77 (H4N6)	35
A/duck/Czechoslovachia/56(H4N6)	34
A/mountain hawk- eagle/Kumamoto/01/07 (H5N1)	29
A/whooper swan/Aomori/1/2008 (H5N1)	37
A/whooper swan/Aomori/2/2008 (H5N1)	36
A/duck/Vietnam/G12/2008 (H5N1)	38
A/chicken/Ibaraki/01/2005 (H5N2)	35
A/E.woodcock/Vietnam /WB8–9/01 (H5N2)	28
A/crow/Kyoto/T2/2004 (H5N1)	41
A/whistling swan/Shimane/499/83(H5N3)	35
A/whistling swan/Shimane/150/2002 (H5N3)	27
A/muscovy duck/Vietnam /G33-TC15&16/2007 (H6N2)	38
A/whistling swan/Shimane/35/80 (H6N3)	33
A/duck/Saga/62/2008 (H7N7)	33
A/quail/Aichi/1/2009 (H7N6)	40
A/whistling swan/Shimane/42/80 (H7N7)	34
A/teal/Tottori/130/2002 (H8N4)	35
A/duck/Hokkaido/26/99 (H9N2)	35
A/whistling swan/Shimane/148/2009 (H9N2)	33
A/chicken/Germany/N/49 (H10N7)	29
A/duck/England/1/56 (H11N6)	33
North American isolate	
A/mallard/NY/6750/78 (H2N2)	35
A/turkey/Massachusetts/65 (H6N2)	36
A/turkey/Ontario/6118/68 (H8N4)	35
A/turkey/Wisconsin/1/66 (H9N2)	35
A/duck/Memphis/546/74 (H11N9)	34
A/mallard duck/Alberta/60/76 (H12N5)	33
A/gull/Maryland/704/77 (H13N6)	31
Non avian influenza virus	
Infectious bronchitis virus strain Beaudette	negative
Influenza B virus strain B/Lee	negative
Newcastle disease virus strain HB-1	negative

Detection time is the average turbidity increment time of the twice replicated experiment.

reaction was carried out for 120 min. Some inhibitors or impurities may have delayed the detection time.

Detection of positive samples by RT-LAMP assay, without the use of a turbidimeter, can be achieved by visual observation of the white precipitate formed by magnesium pyrophosphate in the positive RT-LAMP reaction mixture [12, 13, 25]. Alternatively, RT-LAMP products in a reaction tube can be visualized under ultraviolet light by adding a fluorescence detection reagent to the reaction mixture [10]. In order to confirm whether the primers designed for RT-LAMP are feasible under conditions other than in a turbidimeter, 63°C isothermal conditions were maintained in a conventional water bath and the reaction was carried out for the same reaction time. When observed under a UV lamp at 365 nm, all avian influenza viruses were found to be fluorescent, in contrast to the negative control and influenza B virus strain B/Lee (data not shown). Thus, visual florescence detection assay is compatible with the real-time turbidity detection assay, suggesting that the LAMP system can be performed in a laboratory possessing a simple water bath and a UV light.

In the present study, we developed a rapid and highly sensitive diagnostic system based on RT-LAMP technology to detect the wide range of avian influenza viruses by targeting the matrix gene. Among the 4 sets of the primer, other than that prepared from the North American wild bird group could not detect all the influenza A viruses, or were very slow with regard to turbidity increment time (data not shown). All of the primer sets other than the North American wild bird group had two or more mixed nucleotides in the sequence, as it was not possible to make all primers fall on a conserved region, while in the case of the North American wild bird group primer set, all primers were in the conserved region of the virus sequences within that group.

A spacer comprising several thymidines has been inserted between F1c or B1c and F2 or B2 in other primers (FIP or BIP) [14, 15]. However, the spacer was not used in this study because that LAMP reaction progresses with inner primers lacking spacers, as reported elsewhere [9, 13].

We assessed the sensitivity of the designed primers in the RT-LAMP when detecting avian influenza virus in the tracheal and cloacal swabs, tissues extracts and fecal samples. RT-LAMP was able to detect the avian influenza virus in all specimens within 60 min, except for frozen the fecal sample of the A/whistling swan/Shimane/148/2009. The assay was

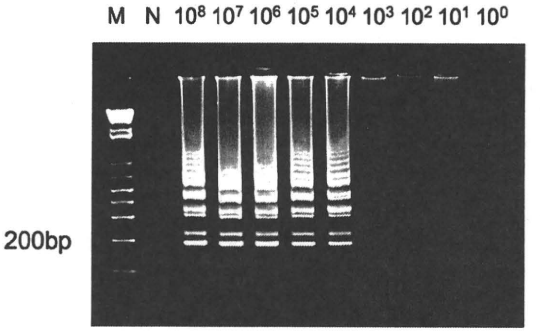

Fig. 1. Detection limit for RT-LAMP assay within a 60-min reaction period using A/chicken/Ibaraki/01/05 viral RNA at a concentration ranging from 10^8 to 10^0 EID_{50}/0.1 m*l*. RT-LAMP products (1 μl) were observed on a 2% agarose gel stained with ethidium bromide. M; DNA marker, NC; Negative control.

Table 3. Detection of avian influenza viruses in swab, tissue and fecal samples by RT-LAMP

Sample	Time of detection
A/mountain hawk-eagle/Kumamoto/1/2007	
Tracheal swab	39[a)]
Cloacal swab	36
Brain tissue	29
Tracheal tissue	39
Muscles	45
A/whooper swan/Aomori/1/2008	
Tracheal swab	50
A/whistling swan/ Shimane/148/09	
Fecal sample	67[b)]
A/duck/Saga/62/08	
Fecal sample	56

a) Time for positive detection (in min).
b) The reaction was carried out for 120 min.

able to detect the $10^{3.5}$ EID_{50} in tracheal swabs and $10^{0.5}$ EID_{50} in the case of muscle tissue extracts among A/mountain hawk-eagle/Kumamoto/01/07-infected tissue samples. As the duck fecal sample infected by A/duck/Saga/62/08 was successfully detected, even with 10-fold diluted RNA samples, the previous results may have been due to the presence of inhibitors, or as a result of some unknown factor that delays detection time. The A/whistling swan/Shimane/148/2009 fecal sample was confirmed to be positive when a separate reaction was carried out for 2 hr. Thus, for fecal samples, the reaction time could be increased in order to avoid the false negative cases.

In conclusion, we report a newly developed RT-LAMP assay with uniquely designed primers that is sensitive and specific to avian influenza viruses, extremely rapid, cost-effective and has potential usefulness in comprehensive avian influenza surveillance and diagnosis.

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