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Short communication

Development of a reverse transcription loop-mediated isothermal amplification assay for the rapid diagnosis of avian influenza A (H7N9) virus infection

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A genetic diagnosis system for detecting avian influenza A (H7N9) virus infection using reverse transcription-loop-mediated isothermal amplification (RT-LAMP) technology was developed. The RT-LAMP assay showed no cross-reactivity with seasonal influenza A (H3N2 and H1N1pdm09) or influenza B viruses circulating in humans or with avian influenza A (H5N1) viruses. The sensitivity of the RT-LAMP assay was 42.47 copies/reaction. Considering the high specificity and sensitivity of the assay for detecting the avian influenza A (H7N9) virus and that the reaction was completed within 30 min, the RT-LAMP assay developed in this study is a promising rapid diagnostic tool for avian influenza A (H7N9) virus infection.

Many subtypes of avian influenza A viruses, including H7N2, H7N3, H9N2, H10N7, and H6N1, infect humans sporadically and cause respiratory illness (Arzey et al., 2012; Reperant et al., 2012; Shi et al., 2013). Cases of humans infected with highly pathogenic avian influenza A (H5N1) viruses have been reported from Asia to Europe and Africa, causing a total of 641 infections and 380 deaths as of October 8, 2013 (http://www.who.int/influenza/human_animal_interface/EN_GIP_20131008CumulativeNumberH5N1cases.pdf). In 2003 in the Netherlands, endemic highly pathogenic avian influenza A (H7N7) virus infections were reported in 90 cases, one of which was fatal (Fouchier et al., 2004; Koopmans et al., 2004).

The first human cases of infection with the avian influenza A (H7N9) virus (A/H7N9) were reported in late March 2013, and the virus was confirmed to have caused 137 infections and 45 deaths in mainland China as of October 25, 2013 (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/10u_ReportWebH7N9Number.pdf) (Gao et al., 2013; Li et al., 2013a). More than 200 human cases have been reported in China within the first 2 months of 2014. Considering that probable human-to-human transmission of the A/H7N9 virus between 2 patients was reported recently and that the airborne transmissibility of the virus between ferrets (a mammalian model) has been reported by several groups, further spread of the infection is of increasing concern (Belser et al., 2013; Qi et al., 2013; Watanabe et al., 2013; Zhang et al., 2013b; Zhu et al., 2013).

A novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), was reported in the early 2000s and the reverse transcription (RT)-LAMP assay can be performed without the need for high-precision instruments and can detect viral genomes within 30 min (Nagamine et al., 2002; Notomi et al., 2000). A/Shanghai/1/2013, A/Shanghai/2/2013, and A/Anhui/1/2013 were isolated from the first three human cases of A/H7N9 infection by using embryonated eggs at the Chinese Center for Disease Control and Prevention, and the sequences of the coding regions of all eight viral genes were deposited quickly in the influenza sequence database of the Global Initiative on Sharing All Influenza Data (GISAID) on March 31, 2013. On the basis of these sequence data, several RT-PCR and RT-LAMP methods targeting A/H7N9 viruses have been developed (Corman et al., 2013; Ge et al., 2013; Hackett et al., 2013; Li et al., 2013b; Nie et al., 2013; Wong et al., 2013; Zhang et al., 2013a) (http://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf). Previously, the direct RT-LAMP method was established to detect viral RNA directly from nasal or nasopharyngeal swabs dispensed in an extraction reagent without an RNA purification step (Nakauchi et al., 2011b). In the present study, with the aim of providing a highly specific and sensitive diagnostic

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Table 1
Primer set.

Primer name	Sequence (5′–3′)	Position[c]	Length (bp)
AH7N9-F3	TTCCTGAGATTCCAAA	995–1010	16
AH7N9-B3	GGTTGGTTTTTTCTATAAGCCG	1177–1198	22
AH7N9-FIP[a]	ACCAACCATCAATTAGGCCTT-	1061–1081 (F1c)	40
(F1c + F2)	CTATTTGGTGCTATAGCGG	1021–1039 (F2)	
AH7N9-BIP[b]	GGTTTCAGACACCAGAATGCACA-	1084–1106 (B1c)	45
(B1c + B2)	CCTGTTATTTGATCAATTGCCG	1145–1166 (B2)	
AH7N9-LF	CCCATCCATTTTCAATGAAAC	1040–1060	21
AH7N9-LB	ACTGCTGCAGATTACAAAAG	1117–1136	20

[a] AH7N9-FIP primer consisted of F1c and F2.
[b] AH7N9-BIP primer consisted of B1c and B2.
[c] The nucleotide positions of the HA gene of A/Anhui/1/2013 are based on the cRNA sequence obtained from the GISAID database (isolate ID number: EPI_ISL_138739).

Table 2
The specificity of the RT-LAMP assay was tested using serial dilutions of viral RNA from H7 subtype avian influenza A viruses.

Virus	Viral RNA concentrations (copies/μL)[a]	Number of positive replicates/number of tests for each assay Dilution rate of viral RNA				
		10^1	10^2	10^3	10^4	10^5
A/Anhui/1/2013 (H7N9)	1.5×10^5	2/2	2/2	2/2	2/2	0/2
A/duck/Fukui/1/2004 (H7N7)	1.5×10^5	2/2	1/2	0/2	0/2	0/2
A/mallard/Netherlands/12/2000 (H7N3)	2.9×10^7	NT	NT	0/2	0/2	0/2
A/duck/Gunma/466/2011 (H7N9)	7.7×10^7	NT	NT	0/2	0/2	0/2
A/duck/Hong Kong/293/1978 (H7N2)	1.4×10^8	NT	NT	0/2	0/2	0/2
A/quail/Aichi/1/2009 (H7N6)	8.2×10^7	NT	NT	0/2	0/2	0/2
A/Netherlands/33/2003 (H7N7)	4.5×10^7	NT	NT	0/2	0/2	0/2

NT indicates "not tested."
[a] Viral RNA copy number was calculated based on the M gene of each virus as described in the text.

tool for the surveillance and early screening of cases infected with the A/H7N9 virus, and specifically for the method to be performed easily in quarantine conditions and clinics should an epidemic of A/H7N9 occur, a new assay was developed to detect the A/H7N9 virus by the RT-LAMP method.

Primer sets for the RT-LAMP assay to detect specifically the HA gene of A/H7N9 were designed based on the HA genes of the A/H7N9 viruses deposited in the influenza sequence database of GISAID using Primer Explorer V4 software (Eiken Chemical, Tokyo, Japan) (Table 1). RT-LAMP was carried out using an RNA Amplification Kit (RT-LAMP; Eiken Chemical). The reaction mixture contained 12.5 μL of 2× reaction mix, 1 μL enzyme mix, 4 μL distilled water, 2.5 μL of 10× primer mix (containing 16 μM each of the FIP and BIP primers, 2 μM each of the F3 and B3 primers, and 8 μM each of the LoopF and LoopB primers), and 5 μL template RNA. The mixture was incubated using a Loopamp Realtime Turbidimeter (LA-320C; Eiken Chemical) for 35 min at 62.5 °C and then for 5 min at 80 °C to terminate the reaction.

Seasonal influenza A and B viruses isolated from humans by using Madin–Darby canine kidney (MDCK) cells, namely, A/Uruguay/716/2007 (H3N2), A/Perth/16/2009 (H3N2), A/Narita/1/2009 (H1N1)pdm09, A/California/07/2009 (H1N1)pdm09, B/Florida/4/2006, B/Brisbane/60/2008, and B/Massachusetts/2/2012, were used to test the specificity of the RT-LAMP assay. The H5 subtype avian influenza A viruses A/chicken/Ibaraki/1/2005 (H5N2) and A/whooper swan/Hokkaido/4/2011 (H5N1) and H7 subtype avian influenza A viruses (listed in Table 2) isolated using embryonated eggs were also used. RNA was prepared from 140 μL of the culture medium of MDCK cells or the allantoic fluid of embryonated eggs using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The yield of the purified viral RNA was checked by performing a type A real-time RT-PCR (rRT-PCR) assay for all viruses, and RNA copy number was calculated based on the M gene, as described previously (Nakauchi et al., 2011a). To evaluate the specificity of the assay, eight nasal swabs suspended in virus transport medium (two nasal swabs confirmed to be positive for seasonal influenza A (H3N2), two positive for influenza A (H1N1pdm09), two positive for influenza B viruses, and two negative for both influenza viruses by real-time RT-PCR) were also used. RNA was extracted from 50 μL of the nasal swabs suspended in virus transport medium using a MagMAX™ 96 Viral Isolation Kit (Ambion, Austin, TX) with KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' instructions. The RT-LAMP assay did not cross-react with seasonal influenza A (H3N2 and H1N1pdm09) or influenza B viruses isolated from humans, with highly pathogenic avian influenza A (H5N1) viruses, or with nasal swabs that were positive for seasonal influenza A (H3N2 or H1N1pdm09) or influenza B, or nasal swabs negative for both influenza viruses (data not shown). The RT-LAMP assay also did not cross-react with five out of six H7 subtype avian influenza A viruses belonging to the Eurasian lineage (Table 2) (Kageyama et al., 2013). The assay reacted slightly with A/duck/Fukui/1/2004 (H7N7); however, its sensitivity was approximately two orders of magnitude lower than that for A/Anhui/1/2013 (Table 2). On the basis of phylogenetic analysis (Takayama et al., under preparation), it was shown that the HA gene of A/duck/Fukui/1/2004 was closest to that of A/Anhui/1/2013 within the six Eurasian lineage H7 subtype avian influenza A viruses used in this study. As the RT-LAMP target region of the HA gene for A/Anhui/1/2013 showed the highest identity with that for A/duck/Fukui/1/2004 (five out of eight regions showed 100% similarity between these viruses, Fig. 1 and Table 2), the RT-LAMP assay may react slightly with A/duck/Fukui/1/2004. Given that the RT-LAMP assay showed little or no cross-reactivity with other Eurasian lineage H7 subtype avian influenza A viruses, the assay developed in this study is highly specific for the detection of the H7 gene of the A/H7N9 virus isolated from humans in China and is able to discriminate the A/H7N9 virus from not only seasonal influenza A and B viruses circulating in humans but also highly pathogenic avian influenza A (H5N1) viruses.

To determine the detection limit of the RT-LAMP assay, RT-PCR was used to amplify the full-length HA gene of A/Anhui/1/2013(H7N9), and the resulting PCR product containing

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