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Short communication

Multiplex RT-PCR detection of H3N2 influenza A virus in dogs

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ABSTRACT

A multiplex RT-PCR (mRT-PCR) assay to detect H3N2 CIV genomic segments was developed as a rapid and cost-effective method. Its performance was evaluated with forty-six influenza A viruses from different hosts using three primer sets which amplify four segments of H3N2 CIV simultaneously. The mRT-PCR has been successful in detecting the viral segments, indicating that it can improve the speed of diagnosis for H3N2 CIV and its reassortants.

Canine influenza virus (CIV) is an RNA virus that causes a highly contagious respiratory disease in dogs [1,2]. Dogs infected with CIV show typical clinical signs of coughing, sneezing, respiratory distress, and fever; however, some occasionally develop secondary complications, such as severe pneumonia [1,2]. To date, two major subtypes of influenza A virus (AIV) in dogs have been identified worldwide. The first strain was isolated from a greyhound in the United States at the beginning of 2004, which was named equine-origin H3N8 influenza virus [3]. The latter strain, isolated from pet dogs in Korea in early 2005, was derived from an avian-origin H3N2 influenza virus [4]. Of the two types, CIVs circulating in Asia have received more attention in recent studies reporting genetic reassortment with pandemic 2009 strains [5–7] and direct transmission from H3N2 human influenza virus [8,9] and H5N1/N2 avian influenza virus [10,11]. The results have supported the fact that dogs have both α-2,6- and α-2,3-sialic acid-linked receptors that bind to AIVs from human and avian hosts, respectively [12]. Thus, dogs have the potential to facilitate interspecies transmission. Previously, H3N2 CIV occurrence was limited to Asian countries, such as Korea, China, and Thailand [2,13–17], whereas H3N8 CIV infections were exclusively found in the United States [18]. However, the latest outbreak of H3N2 CIV was reported in Chicago in the United States in 2015 [19,20], suggesting the global spread of H3N2 CIVs. Despite the rising issues about the recent H3N2 CIVs outbreak, the development of H3N2 CIV-specific primers designed for use with RT-PCR to amplify unique H3N2 CIV genomic segments (i.e., eight genes) has been lacking, compared with attempts to identify other mammalian influenza viruses, including H3N8 CIVs [21–25].

To fill this gap, we developed multiplex RT-PCR assays to amplify each of the four segments of H3N2 CIV in a single reaction, as a cost- and time-effective diagnostic method. To design the primer sets, 695 influenza genomic sequences from different hosts were analyzed to consider genetic differences among hosts, and the specificity of each primer pair was evaluated using the *in silico* method, BLAST, with modified algorithm parameters before empirical testing. Additionally, for more accurate detection of H3N2 CIVs, a DPO (dual priming oligonucleotide) system, an unparalleled primer structure with a polydeoxyinosine (i.e., poly (I)) linker [26], was selectively adopted to block non-specific priming. The diagnostic accuracy of the multiplex RT-PCR with primer sets was assessed using forty-six reference AIVs, including 16 HA subtypes in avian hosts and H1, H2 and H3 subtypes in human, swine, equine, and canine hosts.

We conducted sequence analysis using a subset of the influenza genomic sequences corresponding to Xu [27] NP strain datasets, which provide the discrimination of different host lineages (e.g., human, swine, avian, and equine), along with recent CIV sequences (data not shown). The sequences were downloaded from the IRD (Influenza Research Database) at http://www.fludb.org [28] and were aligned with MAFFT v.7.0 [29] at http://mafft.cbrc.jp/

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Table 1
Reference influenza A viruses tested for multiplex RT-PCR.

	Influenza A virus isolates	Subtype	Year	Host	Source
1	A/Brisbane/59/2007 (H1N1)	H1N1	2007	Human	NCCP
2	A/Brisbane/10/2007 (H3N2)	H3N2	2007		NCCP
3	A/California/7/2009 (pH1N1)	H1N1	2009		NCCP
4	A/Victoria/361/2011 (H3N2)	H3N2	2011		NCCP
5	A/Gyeongnam/4251/2011 (H1N1)	H1N1	2011		NCCP
6	A/Daejeon/56/2011 (H3N2)	H3N2	2011		NCCP
7	A/Jeonbuk/550/2005 (H1N1)	H1N1	2005		NCCP
8	A/Seoul/1304/2004 (H3N2)	H3N2	2004		NCCP
9	A/Korea/01/2009 (pH1N1)	H1N1	2009		KCDC
10	A/swine/Iowa/15/1930 (H1N1)	H1N1	1930	Swine	ATCC
11	A/swine/Iowa/1973 (H1N1)	H1N1	1973		NVSL
12	A/swine/Texas/4199-2/1998 (H3N2)	H3N2	1998		NVSL
13	A/swine/Korea/PZ4/2006 (H1N2)	H1N2	2006		CNU
14	A/swine/Korea/CAS08/2005 (H1N1)	H1N1	2005		CNU
15	A/swine/Korea/CY10/2007 (H3N2)	H3N2	2007		CNU
16	A/swine/Korea/D180-3/2009 (H1N1)	H1N1	2009		QIA
17	A/canine/Korea/BD-1/2013 (H3N2)	H3N2	2013	Canine	QIA
18	A/canine/Korea/CY009/2010 (H3N2)	H3N2	2010		KVCC
19	A/canine/Korea/DG1/2014 (H3N2)	H3N2	2014		QIA
20	A/canine/Korea/01/2010 (H3N1)	H3N1	2010		KRIBB
21	A/canine/Miami/2005 (H3N8)	H3N8	2005		ATCC
22	A/equine/Miami/1/1963 (H3N8)	H3N8	1963	Equine	ATCC
23	A/equine/Alaska/29759/1991 (H3N8)	H3N8	1991		NVSL
24	A/equine/Kentucky/1/1981 (H3N8)	H3N8	1981		NVSL
25	A/equine/Lichtenfeld/1/2012 (H3N8)	H3N8	2012		FUB
26	A/equine/Wildeshausen/2008 (H3N8)	H3N8	2008		FUB
27	A/equine/Richmond/1/07 (H3N8)	H3N8	2007		AHT
28	A/equine/Rio Grande do Sul/1/12 (H3N8)	H3N8	2012		AHT
29	A/equine/Newmarket/2/93 (H3N8)	H3N8	1993		AHT
30	A/equine/South Africa/4/03 (H3N8)	H3N8	2003		AHT
31	A/duck/Kr/U11-1/2007 (H1N2)	H1N2	2007	Avian	QIA
32	A/wild duck/SH38-51/2010 (H2N8)	H2N8	2010		QIA
33	A/duck/Ukraine/1/1963 (H3N8)	H3N8	1963		WHO
34	A/duck/Czechoslovakia/1956 (H4N6)	H4N6	1956		WHO
35	A/duck/Hong Kong/819/1980 (H5N3)	H5N3	1980		WHO
36	A/shearwater/Australia/1/1972 (H6N5)	H6N5	1972		WHO
37	A/duck/Hong Kong/293/1978 (H7N2)	H7N2	1978		WHO
38	A/turkey/Ontario/6118/1968 (H8N4)	H8N4	1968		WHO
39	A/turkey/Wisconsin/1/1966 (H9N2)	H9N2	1966		WHO
40	A/chicken/Germany/n/1949 (H10N7)	H10N7	1949		WHO
41	A/duck/England/1/1956 (H11N6)	H11N6	1956		WHO
42	A/duck/Alberta/60/1976 (H12N5)	H12N5	1976		WHO
43	A/gull/Maryland/704/1977 (H13N6)	H13N6	1977		WHO
44	A/mallard/Gurjev/263/1982 (H14N5)	H14N5	1982		WHO
45	A/shearwater/Australia/2576/1979 (H15N9)	H15N9	1979		WHO
46	A/black-headed gull/Sweden/2/99 (H16N3)	H16N3	1999		EUMCR

The viruses were obtained or purchased from several different sources: NCCP (National Culture Collection for Pathogens, South Korea), KCDC (Korea Center for Disease Control and Prevention, South Korea), ATCC (American Type Culture Collection, USA), NVSL (National Veterinary Services Laboratories, USA), CNU (Dr. Young-Ki Choi, College of Medicine and Medical Research Institute, Chungbuk National University, South Korea), QIA (Animal, Plant and Quarantine Agency, South Korea), KVCC (Korea Veterinary Culture Collection, South Korea), KRIBB (Korea Research Institute of Bioscience & Biotechnology, South Korea), FUB (Dr. Armando Damiani, Free University of Berlin, Germany), AHT (Dr. Debra Elton, Animal Health Trust, Suffolk, United Kingdom), WHO (World Health Organization, Switzerland), and EUMCR (Dr. A.D.M.E Osterhaus, Erasmus University Medical Center Rotterdam, Netherlands).

alignment/server/ using the FFT-NS-2 strategy. As a result, 695 sequences for each segment were used for primer design to investigate the variable and conserved regions among different hosts. The host sequences were as follows: human (254), swine (157), equine (74), canine (32), feline (2), and avian (177).

Primers for multiplex RT-PCR were designed using two applications: primers4clades [30] and MPprimer [31]. To design canine-specific primers among different hosts and optimize PCR conditions for a single reaction, primers4cladeswas used for each segment. The consensus sequences were analyzed in the advanced mode with some parameter changes (i.e., a suitable Tm (55 °C) for the consensus clamp, GTR + G model, and 250–750 amplicons) to determine the optimized primers and PCR regions. Alternatively, MPprimer was employed to search appropriate PCR regions and primer sets for multiplexing RT-PCR. Candidate primer sets from both applications were investigated for comparison, and overlapping or similar PCR regions and primers were selected for *in silico* testing.

Multiple primer candidates suggested by primers4clades and MPprimer were further filtered to increase their specificity and sensitivity using nucleotide blastn v.2.2.31 [32], at http://blast.ncbi.nlm.nih.gov. During the process, the following steps were conducted, as previously described in Ref. [33]. Each primer pair was concatenated with 20 'N's, and BLAST parameters were adjusted in the following manner: nucleotide (nt/nr) database and 'somewhat similar sequences' for the blastn algorithm; word size = 15, expect threshold = 1000; maximum number of target sequences = 500; and the low complexity filter for the algorithm parameters was turned off. The blast results were re-sorted by total score in descending order, and the closest blast hit with avian influenza viruses was summarized in Table 2. Additionally, based on the results, some of the candidate primers that showed more multiple