ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2018 Vol. 28. No. 5. 555~562 DOI : https://doi.org/10.5352/JLS.2018.28.5.555

Rapid Determining for Subtypes and Pandemic Type of Swine Influenza Virus by Diagnostic One-step RT-PCR

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Received January 15, 2018 / Revised May 2, 2018 / Accepted May 21, 2018

Swine influenza virus (SIV) causes one of the most common diseases of the pig population, and its subtypes are determined by hemagglutinin (HA) and neuraminidase (NA). Recently, the SIV subtype diagnosis has been developed. The method using antigen-antibody reaction rather than PCR was mainly used because of the large change in the ribonucleotide sequences of SIV. Here, we have developed 10 diagnostic primer sets through multi-nucleotide sequences alignment of spreaded SIV since 2008 in Korea and then optimized the reaction of the one-step RT-PCR for rapid determination of SIV subtype. In addition, specific primers were designed to early determine the pandemic SIV by detecting unique M sequences proven in highly infectious and virulent subtypes of the influenza H1N1 (pH1N1). Here, some of the SIVs spread in Korea from 2008 to 2014 have been tested to determine the subtypes and pandemic potential of SIV. All diagnostic primer sets were found to be able to accurately determine the SIV subtype and to detect the pandemic SIV. In conclusion, it was confirmed that the optimized one-step RT-PCR analysis using these primer sets is useful for rapid diagnosis of SIV subtypes. These results can be used for development of SIV subtype diagnostic kit to early detect before virulent SIV spreads do.

Key words : Diagnostic one-step RT-PCR, pandemic H1N1, swine influenza virus

Introduction

Swine influenza virus belongs to orthomyxoviridae and influenza A virus, and is divided into subtypes based on two proteins HA (H1-H16) and NA (N1-N9) on the virus surface [22]. SIV causes one of the common respiratory illnesses in pig populations. These can quickly spread to the world and have a great impact on the socioeconomic environment [18]. In 2009, there was a pandemic H1N1 virus resulting from antigenic shift between animal and human origin of SIV [2, 12, 14, 22]. It spread throughout the world and infected some 500,000 people [5, 12, 24]. Therefore, rapid and sensitive detection techniques are needed to control epidemic and epidemic diseases. Conventional subtype diagnostic methods use the rapid influenza diagnostic test (RIDT) based on immunochromatography to detect viral antigens. This is considered to be the best and quickest diagnostic method for influenza virus diagnosis [13]. However, this method has difficulties in classifying influenza A subtypes and has been performed mainly for classification of influenza A and B virus types because of low sensitivity and requirement of high amount of SIV [1, 7, 20].

Therefore, reverse transcriptase chain reaction (RT-PCR) techniques that sensitively detect influenza virus have been developed in recent years [3, 4, 15, 21]. PCR-based diagnostic methods can detect type A influenza viruses using highly specific primers and can distinguish other subtypes of influenza A virus [8, 16]. Recently, M protein has been focused as a target protein to discriminate between pandemic or endemic SIV. However, pandemic SIV determination by the method based on antibody-antigen response is quite difficult

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because M2 protein is pore protein, which is less exposed than HA and NA proteins out of the viral surface. Therefore, the detection method for particular pandemic M sequence by RT-PCR and one-step RT-PCR is developed together with designed specific primers for M sequence [9, 10, 16].

The aim of this study is to rapidly diagnose SIV subtypes and pandemic SIV using specific primers and an optimized one-step RT-PCR analysis system. We designed 10 diagnostic primer sets to identify the types of SIV using multiple nucleotide sequence alignment of SIV appeared from 2008 to 2014. We have also successfully determined the virulent subtype pH1N1 of the SIV by detecting a unique M sequence. Ultimately, we are aiming at early detection of pre-spread SIV by developing step RT-PCR based on unique fragments of the HA, NA and M2 genes of influenza A virus.

Materials and Methods

Viral RNA and primers

Viral RNAs (Table 1) were provided and tested at the Animal and Plant Quarantine Agency (APQA) and stored in -80℃ until synthesis cDNA. Those 9 strains of SIV subtypes were named by APQA as follows; D180, 251-3 and Seoul (pandermic) for H1N1 strains, G63, 103 for H1N2 strains, A81, BRH14, G47, A18 (pandermic) for H3N2. The primers for subtyping of SIV were designed by multiple alignment of HA, NA and M gene sequences. The alignment was performed using by ClustalW2 (http://www.genome.jp/tools/clustalw/) with SIV nucleotide sequences from the Influenza Research Database (http://www.fludb.org/).

cDNA synthesis and RT-PCR

Reverse transcription (RT) for complementary DNA synthesis was performed using iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA) according to the manufacturer's instructions. SIVs were subtyped by conventional RT-PCR targeting the HA, NA and M gene using TaKaRa Ex Taq™ (TaKaRa, Otsu, Japan) with 2 ng of SIV RNA templates. Conventional RT-PCR cycling conditions were as follows: pre melting for 5 minutes at 94℃; 35 cycles of denaturation for 30 seconds at 94℃, annealing for 30 seconds 53~56℃ and extension for 30 seconds at 72℃; and a final extension of 5 minutes at 72℃.

One step RT-PCR for virus subtyping

One-step RT-PCR targeting the HA, NA and M gene was performed using QIAGEN® One-Step RT-PCR Kit (Qiagen, Hilden, Germany). The PCR mixture consists of QIAGEN One-Step RT-PCR 5 x buffer 10 μl, 2 μl of QIAGEN One-Step RT-PCR Enzyme mix, 2 μl of dNTP Mix, 5 pmole each primer, 2 ng of RNA templates and RNase-free water. Cycling conditions for one step RT-PCR were as follows: reverse transcription for 30 minutes at 50℃, initial PCR activation for 15 minutes at 95℃; 35 cycles of denaturation for 30 seconds at 94℃, annealing for 30 seconds 50~68℃ and extension for 60 seconds at 72℃; and a final extension of 10 minutes at 72℃. In this study, diagnostic One-Step RT-PCR with modified cycling condition was also developed and performed with constructing a mixture of Labopass™ M-MuLV reverse transcriptase and Labopass™ G-Taq polymerase from Cosmo genetech (Korea) with optimized buffer.

Results

Construction of specific primers for HA and NA gene of Swine Influenza A Virus

In order to develop primer set for subtyping of SIVs, we multiple-aligned with complete nucleotide sequences of

Table 1. SIV strain name and suggested subtypes used in this study

Pre-determined subtype	Strain name[1)]	Strain full name
H1N1	D180	A/swine/Korea/VDS3/2009
H1N1	251-3	A/swine/Korea/VDS4/2008
H1N1	Seoul (pandemic)[2)]	A/swine/Korea/VD1/2009
H1N2	G63	A/swine/Korea/VDS1/2010
H1N2	103	A/swine/Korea/103/2009
H3N2	A81	A/swine/Korea/A81/2013
H3N2	BRH14	A/swine/Korea/BRH14/2013
H3N2	G47	A/swine/Korea/G47/2013
H3N2	A18 (pandemic)	A/swine/Korea/A18/2011

[1)]The strains of SIV were named and pre-determined those subtypes by APQA (Animal and Plant Quarantine Agency, South Korea).
[2)]The strain 'Seoul' was used in this study with the isolated and tested strain by APQA, Korea.

H1N1, H3N1, H1N2, H3N2 swine influenza A virus and pandemic pH1N1 SIV that occurred from 2008 to 2014 in South Korea (Table 2). As a result, we found specifically conserved sequences among SIV subtypes and developed complementary primer sets. In order to determine the H subtypes, we aligned and compared HA sequences in H1N1 and H1N2 in one set and H3N1 & H3N2 SIVs in another set and then constructed H1 primers, H1_409, H1_822 and H3 primer, H3_278, H3_781. The NA sequences of H1N1 & H3N1 and H1N2 & H3N2 SIVs also compared in the same manner to design N1 primers, N1_399 and N1_461 and N2 primers, N2_450 and N2_544. Finally, to determine pandemic pH1N1 A virus, we multiple aligned the matrix M gene sequences and identified specific conserved sequences. And then the detection primers, pH1N1_ 481 and pH1N1_846, were developed (Table 3).

Subtyping and pandemicity identification of Swine Influenza A Virus spread in Korea

By multiple sequences alignment, we designed 4 sets of primers which were able to discriminate H1 and H3 subtype. Two specific H1 primers, H1_409, H1_822, were clearly used for detection for H1N1 and H1N2 subtypes of SIV named D180, 251-3, Seoul and 103 which had been occurred in South Korea. Also, SIVs named A81, BRH14, G47 and A18 were determined as H3 subtype by using two H3 primers, H3_278, H3_781 (Fig. 1A, Fig. 1B). But SIV named G63 was able to be determined as a H1 subtype by showing a weak band in H1 detection PCR using only with H1_409 primer (Fig. 1A). In the same manner, the N1 and N2 forms of all SIVs were distinguished by RT-PCR with four sets of NA primers. N1 specific band sizes of 399 bp and 461 bp were confirmed by PCR reactions on the SIV N1 subtype using

Table 2 GeneBank accession numbers of SIVs and segments lengths of each target genes

Subtype	Strain name	NCBI Taxon. ID #	GeneBank accession # / Segment length (bp)		
			HA	NA	M
H1N1	A/swine/Korea/CY01-04/2012	1281708	KC471369/1701	KC471371/1410	KC471372/982
	A/swine/Korea/CY01-05/2012	1281709	KC471377/1701	KC471379/1410	KC471380/982
	A/swine/Korea/CY01-06/2012	1281710	KC471385/1701	KC471387/1410	KC471388/982
	A/swine/Korea/CY11-01/2011(H1N1)	1281711	KC471345/1701	KC471347/1410	KC471348/982
	A/swine/Korea/CY11-02/2011(H1N1)	1281712	KC471353/1701	KC471355/1410	KC471356/982
	A/swine/Korea/SCJ28/2010(H1N1)	762450	HM189557/1701	HM189445/1410	HM189475/977
	A/swine/Korea/SCJ33/2010(H1N1)	762451	HM189558/1701	HM189446/1410	HM189476/977
	A/swine/Korea/SCJ41/2010(H1N1)	762452	HM189559/1701	HM189447/1410	HM189477/977
	A/swine/Korea/SCJ42/2010(H1N1)	762453	HM189560/1701	HM186448/1410	HM189478/976
H1N2	A/swine/Korea/CY03-11/2012(H1N2)	1281717	KC471425/1701	KC471427/410	KC471428/982
	A/swine/Korea/CY0423-12/2013	1337100	KF142495/1778	KF142497/1466	KF142498/1027
	A/swine/Korea/CY0423-33/2013(H1N2)	1337101	KF142503/1732	KF142505/1410	KF142506/2007
	A/swine/Korea/CY12-03/2011(H1N2)	1281718	KC471361/1701	KC471363/410	KC471364/982
	A/swine/Korea/VDS1/2010	1038687	JN043428/1701	JN043437/1410	JN043433/971
H3N1	A/swine/Korea/CY02-07/2012	1281713	KC471393/1701	KC471395/1410	KC471396/982
	A/swine/Korea/CY02-08/2012(H3N1)	1281714	KC471401/1701	KC471403/1410	KC471404/982
	A/swine/Korea/CY03-12/2012(H3N1)	1281715	KC471433/1701	KC471435/1410	KC47136/982
	A/swine/Korea/CY03-13/2012(H3N1)	1281716	KC471441/1701	KC471443/1410	KC47144/982
H3N2	A/swine/Korea/A18/2011	1224818	JX501999/1701	JX502005/1410	JX502002/982
	A/swine/Korea/CY02-09/2012(H3N2)	1281719	KC471409/1701	KC471411/1410	KC471412/982
	A/swine/Korea/CY02-10/2012(H3N2)	1281720	KC471417/1701	KC471419/1410	KC471420/982
	A/swine/Korea/CY03-14/2012(H3N2)	1281721	KC471449/1701	KC471451/1410	KC471452/982
	A/swine/Korea/CY03-15/2012(H3N2)	1281722	KC471457/1701	KC471459/1410	KC471460/982
	A/swine/Korea/CY03-16/2012(H3N2)	1281723	KC471465/1701	KC471467/1410	KC471468/982
	A/swine/Korea/CY03-17/2012(H3N2)	1281724	KC471473/1701	KC471475/1410	KC471476/982
	A/swine/Korea/CY03-18/2012(H3N2)	1281725	KC471481/1701	KC471483/1410	KC471484/982
	A/swine/Korea/CY03-19/2012(H3N2)	1281726	KC471489/1701	KC471491/1410	KC471492/982
	A/swine/Korea/D79/2011	1224819	JX502000/1701	JX502006/1410	JX502003/982
	A/swine/Korea/KSB/2012	1224820	JX501998/1701	JX502004/1410	JX502001/982
	A/swine/Korea/PL01/2012	1357697	KF382729/1701	KF382731/1410	KF382732/982

Table 3. List of primers used in this study

Primers	Target gene	Targeted subtype	Oligonucleotide sequences (5′- 3′)	Product size (bp)
SIV_H1_409	HA	H1	F : ACACAGTACGCAGCAGTAG R : ATGAACTGGCGACAGTTG	409
SIV_H1_822	HA	H1	F : GGTACCGAGATACGCATTCGC R : GAACTGGCGACAGTTGAATAG	822
SIV_H3_278	HA	H3	F : TGGTCCAGAGTTCCTCAACA R : CCGTCTTGAGCAACTCCAGT	278
SIV_H3_781	HA	H3	F : GTCCAAGCATCAGGGAGAG R : GCAGTTGCCTTCTTGTTC	781
SIV_N1_399	NA	N1	F : ATCGACCGTGGGTGTCTTTC R : CTTTGGGTCGCCCTCTGATT	399
SIV_N1_461	NA	N1	F : AGCTGTCCTATTGGTGAAGT R : TGATATTCCAGATTCTGGTT	461
SIV_N2_450	NA	N2	F : CGAACCCTATTGATGAATGA R : TATATCTACGATGGGCCT	450
SIV_N2_544	NA	N2	F : ATTACAGGATTTGCACC R : ACATGCTGAGCACTTC	544
SIV_pHIN1_481	M	Pandemic H1N1	F : GACCACAGAAGCTGCCTT R : GGCACTCCTTCCGTAGAAG	481
SIV_pHIN1_846	M	Pandemic H1N1	F : GCGCAGAGACTGGAAAG R : GATATTCTTCCCTCATGGA	846

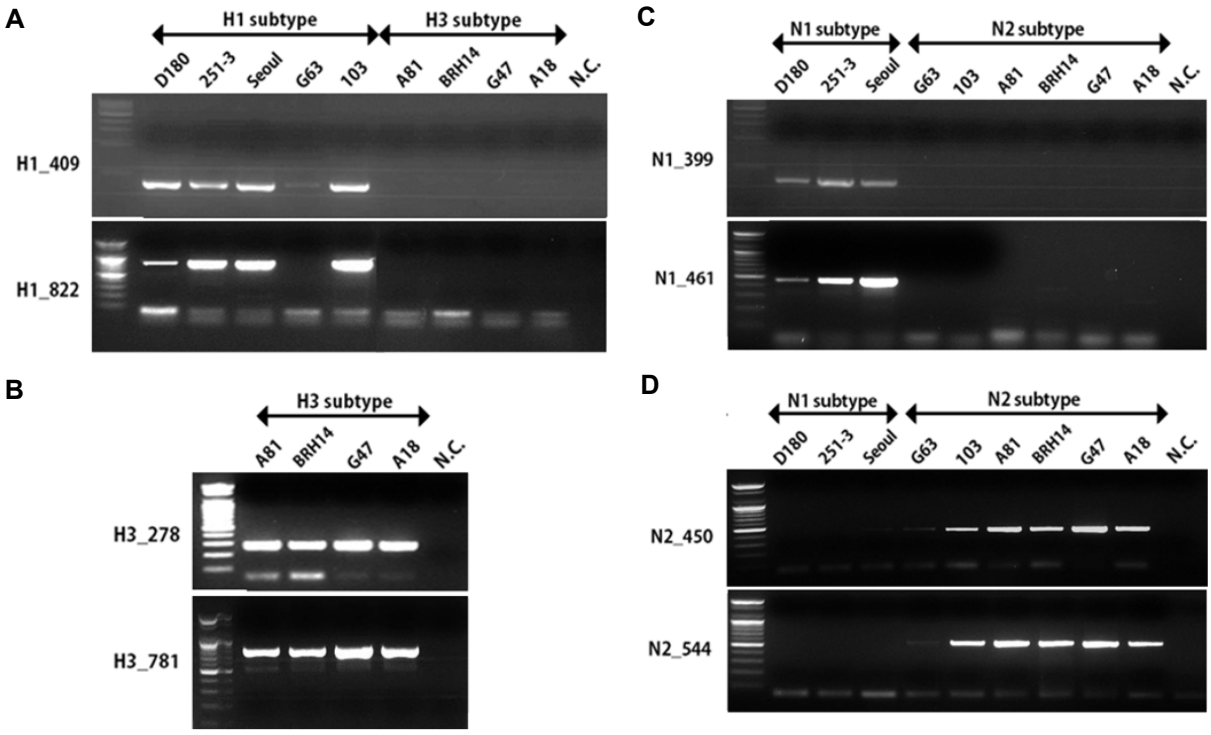

Fig. 1. Determination of HA and NA type for SIV subtyping by conventional PCR. Subtyping for SIV HA and NA by PCR amplification with designed primers. A, Conventional PCR was performed with specific H1 primer sets, H1-409, H1_822, based on conserved sequence. All H1 subtype of SIV was determined by PCR except strain G63. B, The H3 subtypes of SIV were also determined by PCR with designed H3 primers, H3_278 and H3_781, without any misidentification. Also, PCR amplifications for NA subtyping were performed with N1 and N2 primers. C, Conventional PCR for N1 subtying was performed with specific N1 primer sets, N1_399 and N1_461 based on conserved sequence. d, N2 subtype of SIVs was determined by using N2 primer, N2_450 and N2_544. Negative control (N.C.) indicates the result of PCR reaction without template.

primers N1_399 and N1_461 designed through N1 subtype conserved sequence analysis. Also, determination PCR using N2 subtype-specific N2_450 and N2_544 primers revealed specific bands representing for N2 subtype (Fig. 1C, Fig. 1D).

In the case of G63 subtype determination, a weak N2 subtype-specific band was observed in NA subtyping PCR as seen in H1 subtype determination (Fig. 1D). As indicated by the weak band in N1 detection PCR using both N1_399 and N1_461 primers, the SIV named D180 was determined as N1. However, these results demonstrate that the designed HA and NA subtype specific primers are suitable for the diagnosis of SIV subtypes.

In addition, to determine the virulent viruses called pH1N1, we used specifically designed primers based on highly conserved matrix M gene among in pandemic SIV subtypes. Apparently, PCR diagnoses using two designed primers, pH1N1_481 and pH1N1_846 were clearly distinguished the pH1N1 viruses from other SIVs (Fig. 2A).

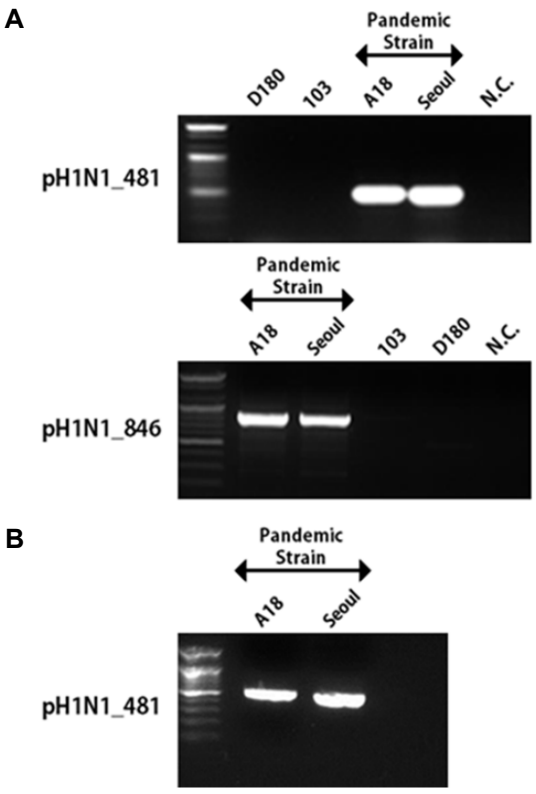

Fig. 2. Identification of pandemic SIV strain. A, Conventional PCR was performed with specific primer sets based on conserved sequence of pandemic strain. The pandemic SIV were amplified by selected primers, pH1N1_481 and pH1N1_846. B, The pandemic strain were determined without mis-detection by One-step RT-PCR with specific primer, pH1N1_481.

Rapid determination of SIV subtypes by developed One-step RT-PCR

We conducted a diagnostic PCR using the QIA One-Step RT-PCR Kit and sensitive primers selected based on previous results using conventional PCR. All viruses were clearly identified by the marked bands as HA and NA subtypes and then pandemic SIV also was distinguished from other SIVs (Fig. 3, Fig. 2B). However, as in the previous two-step RT-PCR, the SIV of D180 was insufficient to determine the subtype. Therefore, we have also developed one-step RT-PCR with optimized buffering components. Here, the most sensitive and specific primers identified by conventional RT-PCR methods were selected for diagnostic one-step RT-PCR analysis (Fig. 4). However, in the H1N2 SIV, G63, which has never been reported as an epidemic, this diagnostic PCR reaction shows a specially amplified band that is pandemic. In addition, one-step RT-PCR with BRH14, G47 and A81 identified as H3N2 also showed that these SIVs

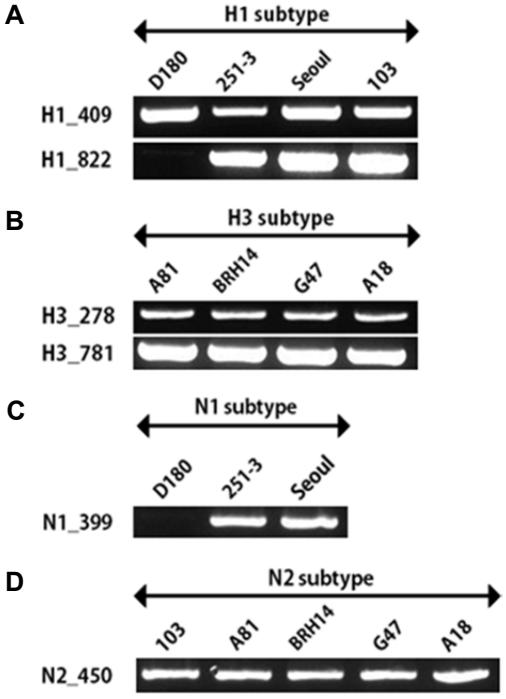

Fig. 3. Identification of SIV subtype by One-step RT-PCR with selected primers. SIV subtyping with amplification of HA and NA genes by One-step RT-PCR. A, B, One-step RT-PCR was performed with H1 subtype-specifically designed primers, H1_409 and H1_822 and with H3 specific primer sets, H3_278 and H3_781. c, d, The SIV contained N1, N2 were amplified and determined using primers, N1_399 and N2_450. However, strain 'D180, determined as H1N1 by conventional PCR was not detected by selected primers H1_822 and N1_399.

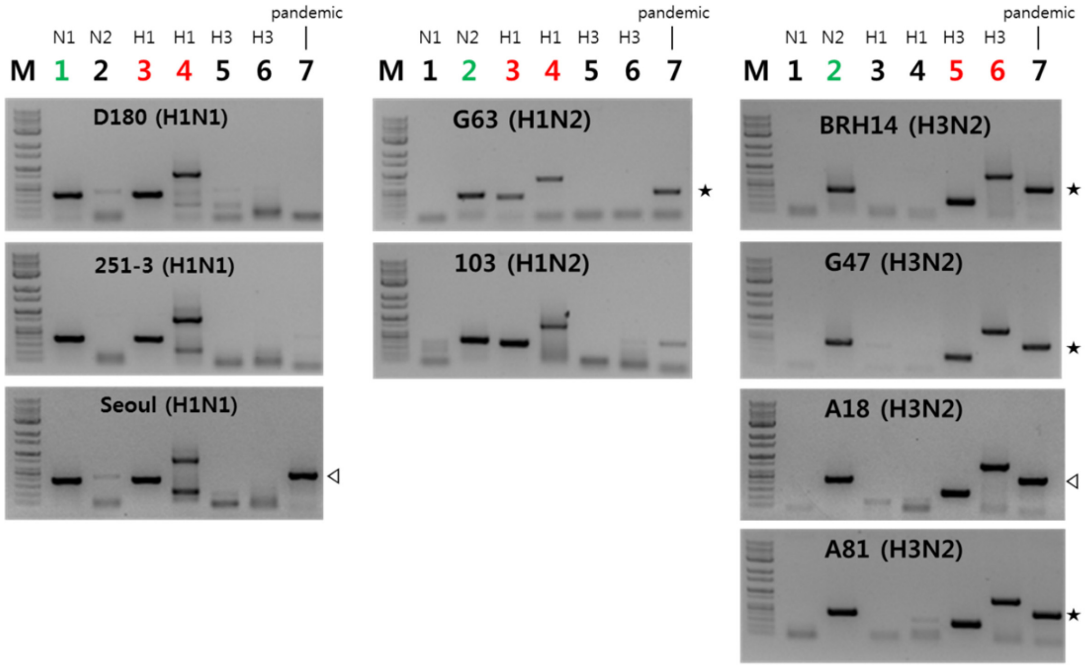

Fig. 4. Diagnosis of pandemic strain and identification of subtypes from SIV by One-step RT-PCR with specific primer sets. Analysis of SIV for subtyping and diagnosis of pandemic strain was performed by modified One-step RT-PCR using optimized buffers and optimized conditions. All primer sets were well-constructed for determination of SIV subtypes corresponding to specific amplification. However, PCR reaction for pandemic SIV determination with primer 7 showed exact PCR band even in three out of seven SIVs known as non-pandemic. (M: size marker, 1: N1_399, 2: N2_450, 3: H1_409, 4: H1_822, 5: H3_278, 6: H3_781, 7: pH1N1M_481, open triangle: pandemic strain, asterisk: nonspecific band, see Discussion).

had an M protein with pandemic features.

As a result of the one-step RT-PCR developed, all SIVs spread in Korea have been identified as precise subtypes such as H1N1, H1N2, H3N1 and H3N2, and have distinguished pandemic SIVs with specific M proteins. Also subtyping for SIV G63 and D180 were determined to its corresponding subtype showing high band intensities. Overall, these results, using the developed diagnostic one-step RT-PCR analysis, suggest that spread SIV can be rapidly identified as a subtype of the SIV, as well as identifying potential pandemicity.

Discussion

The emergence of novel influenza virus is occurred by genetic reassortment among different influenza viruses co-infection in the same host or mutative variation of HA and NA gene [3]. Occasionally, it may lead to high infective virus such as pandemic H1N1 in 2009. The pH1N1 virus is representative fatal influenza virus having mixed genomes of swine, avian and human influenza virus. In particular, it is characterized by being easily transmitted from pig to pig [4, 8, 16]. In order to prevent the spread of pandemic or endemic viruses, the important point is timely diagnosis and treatment. Generally, RT-PCR based on differences among nucleotide sequences and RIDT based on antigenic detection are highly sensitive and specific methods [1, 6, 11]. Recently, quantitative RT-PCR assays are mostly used for rapid detection of influenza. Comparing these two methods, RIDT is less sensitive than RT-PCR assay [15, 17, 19, 23]. On the other hand, for the detecting subtype signature by PCR method, target conserved sequences of HA, NA and M genes are required to develop the specific primers by multiple alignments of full genome sequences [6, 9, 10, 16].

In this study, we aimed to develop the primer sets and one-step RT-PCR assay method for rapidly and specifically determining subtypes of Swine Influenza A viruses. First, conventional two-step RT-PCR was performed, and the subtype of SIV was determined by electrophoresis. With the exception of only one SIV 'G63' showing a low intensity band in the H1 subtyping, subtypes of other SIVs are clearly distinguishable by designed primers. In this regard, there are possibilities that 'G63' has a large numbers of variations in nucleotide sequences or 'G63 is a pandemic virus but is not

proved as an epidemic because of efficient prevention control. Second, the primers for SIV subtype diagnosis used by selecting from the results of conventional RT-PCR might be very sensitive in one-step RT-PCR assay. However, G63, BRH14, G47 and A81 were recently proved to have a M protein derived from pandemic H1N1(unpublished data). As a result, all samples are determined clearly. In other words, developed one-step RT-PCR analysis system using optimized the buffer solution and the developed primer sets was shown to be highly efficient at accurately detecting SIV, determining subtypes, and distinguishing the pandemicity. Taken together, the developed and optimized one-step RT-PCR using the specific primers described above makes it possible to diagnose the subtypes and pathogenicity of SIV. Also, this efficient diagnostic assay can be assisted to detect SIV subtypes rapidly and prevent the virulent SIV infection.

Acknowledgment

This work supported by a grant from 2015 Research Funds of Andong National University.

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초록 : 진단용 one-step RT-PCR을 통한 돼지 인플루엔자 바이러스의 아형 및 pandemic 유형에 대한 신속한 결정

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Swine influenza virus (SIV)는 돼지 개체군에서 가장 흔한 질병을 일으키는 바이러스 중 하나이며 그 subtype은 hemagglutinin (HA)와 neuraminidase (NA)에 의해 결정됩니다. 최근 SIV subtype 진단 방법이 개발되고 있으나 SIV의 리보뉴클레오타이드 서열의 많은 변이로 인해 PCR 보다는 항원-항체 반응을 이용하는 방법이 주로 사용되고 있다. 본 연구에서는 SIV 하위 유형의 신속한 결정을 위하여 2008년 이후 국내에서 발생한 SIV의 다중 염기서열 정렬을 통하여 10개의 subtype 진단 프라이머 세트를 개발하고 이를 이용한 one-step RT-PCR 반응을 최적화하였다. 또한 감염력이 높고 독성이 있는 인플루엔자 H1N1 (pH1N1)의 아형에서 확인된 독특한 M 유전자 서열을 검출함으로써 pandemic SIV를 조기에 결정하도록 특이적 프라이머를 설계하였다. 2008년부터 2014년까지 한국에서 발생한 9종의 SIV RNA를 활용하여 SIV의 아형 및 pandemic 가능성을 결정하기 위해 시험 분석한 결과 모든 진단 프라이머 세트는 SIV 아형을 정확하게 결정하였으며 pandemic SIV를 검출할 수 있는 것으로 확인되었다. 결과적으로 이들 프라이머 세트를 이용한 최적화된 one-step RT-PCR 분석이 SIV 아형의 신속한 진단에 유용하다는 것이 확인하였다. 이러한 결과는 SIV 하위 유형 및 pandemic SIV가 확산되기 전에 조기 발견을 위한 키트로 개발될 수 있음을 시사한다.