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Full length sequencing of all nine subtypes of the neuraminidase gene of influenza A viruses using subtype specific primer sets

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Abstract

An RT-PCR based method was developed using subtype specific overlapping primers to obtain full length amplification of neuraminidase (NA) gene from all subtypes (N1-N9) of influenza A viruses. This method was validated using reference strains of avian influenza viruses (AIV) (N1-N9), human influenza viruses (N1and N2), and swine influenza viruses (N1-N3). Amplification of the NA gene was obtained with all viruses tested. Additionally, 200 field isolates of AIV from wild birds were tested by this method and the NA gene was amplified in all isolates. The NA subtype of all 200 isolates was determined by further sequencing of the amplified NA genes and all sequences were submitted to GenBank. The method described in this paper can be used to determine subtype of influenza isolates as well as their evolution and mutations if any, in the NA gene.

Keywords

Antiviral resistance; Avian influenza virus; Gene amplification; Influenza A virus; Neuraminidase gene; Reverse transcription-polymerase chain reaction (RT-PCR); Subtyping

1. Introduction

Influenza A viruses are important pathogens of humans and animals. The last few years have witnessed an increase in the number of outbreaks due to influenza A viruses (Capua and Alexander, 2007), which are subtyped on the basis of antigenic and/or genetic differences in their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) (Fereidouni et al., 2009). Currently 16 subtypes of HA (H1–16) and 9 subtypes of NA (N1–9) have been identified (Fouchier et al., 2005).

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Widespread outbreaks caused by avian, swine, and human influenza viruses necessitate the development of simple and reliable methods for virus detection and identification. Virus isolation in cell cultures or embryonated chicken eggs followed by heamagglutinin inhibition (HI) and neuraminidase inhibition (NI) assays (Allan and Gough, 1974; Aymard-Henry et al., 1973; Van Deusen et al., 1984) for viral subtyping are standard diagnostic methods. Molecular methods such as reverse-transcriptase polymerase chain reaction (RT-PCR), probe hybridization, and microarray are also considered sensitive and specific methods for the identification and subtyping influenza viruses (Pasick, 2008; Zou, 1997).

Although, many RT-PCR based methods have been developed for subtyping of influenza A viruses (Hoffmann et al., 2001; Fereidouni et al., 2009; Jindal et al., 2009), most of these methods have been optimized only for certain specific subtypes of influenza A viruses or for amplification of the HA gene only (Stockton et al., 1998; Takao et al., 2002; Spackman et al., 2002). Recently, development of RT-PCR methods for subyping of the NA gene have also been reported (Alvarez et al., 2008; Fereidouni et al. 2009; Qiu et al., 2009). However, in most of these studies, only a small portion of the NA gene was targeted for amplification and reports on full length amplification of the NA gene by RT-PCR method are meager. Hoffmann et al. (2001) reported on the use of five sets of primers for full length amplification NA gene from all N- types of IAV. More recently, Jindal et al. (2009) reported on the use of a set of degenerate primers for full length amplification of four genes (HA, NA, matrix, and non-structural protein) of influenza A viruses. Similarly, Kreibich et al. (2009) developed a PCR method for the full length amplification of four genes (NA, NP, M, and NS) of influenza in a single reaction.

However, full length amplification is not always amenable to direct sequencing because of the large size of the NA gene (approx 1400 bp). Reliable sequencing of NA gene is needed to determine the susceptibility of the isolates to antiviral drugs and for phylogenetic analysis. For full length amplification of NA gene from all subtypes of influenza A viruses, subtype specific primers were used and all 9 NA subtype were amplified in short overlapping sequences, which were later aligned together using Sequencher software (ver. 4.8) to generate full length sequence of the NA gene.

2. Materials and methods

2.1. Viruses

For the development and validation of this method, RNA extracts from known IAV isolates representing all NA subtypes (N1-N9) were obtained from the National Veterinary Services Laboratory (NVSL), Ames, IA (Table 1). In addition, field isolates (n=200) of avian influenza viruses (AIV) isolated from wild waterfowl at the University of Georgia, Athens, GA were used.

2.2. Primer design

Details of the primers used in this study are given in Table 2. Primers were designed by using DNAstar software (www.msi.umn.edu) based on sequences available in public domain (www.ncbi.nlm.nih.gov). For each subtype, up to four sets of primers were designed for

full length amplification of the NA gene. Only those primer pairs were selected which were specific to each NA subtype and hybridized to all published sequences of a given subtype. In addition, primers of Hoffman et al. (2001) for the amplification of NA gene, were also used.

2.3. Amplification of NA gene

2.3.1. Reference strains: Since NA type of reference strains was known, RT-PCR was performed using subtype specific primers. RT-PCR was performed using one step RT-PCR kit (Qiagen, Valencia, CA). The reaction mixture consisted of 10μl of 5x RT-PCR buffer, 400 μM of each dNTPs, 2μl of enzyme mix, 0.6 μM of each forward and reverse primers, 100 ng of template, and RNAase free water to make a total volume of 50 μl. Amplification involved an initial reverse transcription step at 50° C for 30 min followed by initial denaturation at 95° C for 15 min, 35 cycles of denaturation at 94° C for 1 min, annealing at 53° C for 1 min and extension at 72° C for 1 min, and a final extension step at 72° C for 10 min.

2.3.2. Field isolates: Since all field isolates were of unknown subtype, RT-PCR was performed with Hoffman et al (2001) primers followed by sequencing. Sequences thus obtained were aligned with existing database (BLAST, www.ncbi.nlm.nih.gov) to determine viral subtype. After determining the subtype, each isolate was subjected to RT-PCR using subtype specific primers (Table 1). RT-PCR conditions used for both of these steps were the same as described above.

2.4. Sequencing

For sequencing, PCR amplicons were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and purified DNA fragments were submitted to the DNA sequencing and Analysis Facility, Biomedical Genomics Center, University of Minnesota. Sequencing was done in both directions (forward and reverse) using the same primers as used for amplification. To obtain full length sequence, a subset of sequences for a given isolate was aligned using Sequencer software (ver. 4.8) (www.msi.umn.edu).

3. Results

To develop an RT-PCR method for full length amplification of NA gene from all influenza A subtypes (N1–9), subtype specific primers were designed based on the sequences available in GenBank. These primers and those described by Hoffmann et al (2001), were used to obtain full length sequence of the NA gene. For each subtype, up to four sets of overlapping primers were designed and each primer set amplified a region of about 500 bp. RT-PCR was carried out with RNA extracts of viral isolates representing all nine NA subtypes of influenza A viruses. The RT-PCR conditions were so optimized that same reaction conditions could be used for the amplification of NA gene from all subtypes. The primer sets designed in this study were found to be specific since no non-specific PCR products were observed on agarose gel. Using these universal reaction conditions, amplification was achieved with all primer pairs.

To determine the sensitivity of primers, 10-fold serial dilutions (up to 1:10,000) of RNA extracts were tested with subtype specific primers designed in this study. For all subtypes,

except for N7, the sensitivity of primers was found to be up to 1:10,000. For N7 primers, the sensitivity was found to be 1:100. When the same dilutions were tested with primers described by Hoffman et al. (2001), the sensitivity was found to be between 1:1,000 and 1:10,000. However, some non-specific amplifications were also observed with the Hoffman primers. All primers designed in this study were found to be specific for their respective subtypes as no cross reactivity with any other subtype was observed. Primers described by Hoffman et al. (2001) were also found to be specific.

The usefulness of these primers and protocol for NA subtyping was assessed using 200 AI viruses isolated from wild birds. Using this protocol, amplification was achieved for all 200 isolates and all could be subtyped successfully. Details of subtyping results are given in Table 3. Full length sequences of NA gene from all isolates were obtained by aligning the respective overlapping sequences and all sequences were submitted to the GenBank (Table 3). The primers were also found to provide full length amplification of the NA gene of human and swine influenza isolates (Table 1).

4. Discussion

PCR based methods are the most sensitive and specific methods for rapid and accurate subtyping of influenza A viruses (Schweiger et al., 2001; Lee et al., 2001; Hindiyeh et al., 2005). However, most of the methods which are available for NA typing have been standardized to amplify only a short region of the NA gene. Full length sequence of NA gene is important as this allows not only the NA-typing but can also be useful for further studies such as phylogenetic analysis or mutational analysis for antiviral resistance.

The purpose of this study was to develop a specific and sensitive RT-PCR method for full length amplification of NA genes from all nine subtypes of influenza A viruses. To do this,, the NA gene was amplified in short overlapping fragments (about 500 bp long) using subtype specific primers and then these fragments were aligned together to generate full length sequence. This approach is preferred over direct sequencing because it not only allows subtyping, but also generates accurate sequences that can be used for phylogenetic analysis or mutational analysis. So far there has been only one published report (Obenauer et al., 2006) on the use of this approach for full length amplification of NA gene from all subtypes. However, in this study more than 200 sets of NA primers were used for full length sequencing of NA gene from all subtypes of AIV whereas in the present study, only five sets of primers per subtype (except for N9 for which 6 sets of primers were used) were used to achieve full length sequencing of NA gene with high accuracy. Another advantage of this method is that the same reaction conditions can be used for all RT-PCRs.

This method was found to be highly specific (100%) and useful to amplify NA gene from all nine reference strains and 200 field isolates of avian origin. This is in contrast to RT-PCR methods developed by other workers. For example, Alvarez et al. (2008) reported a touchdown one step RT-PCR method using a universal primer set for detecting all 9 NA subtypes with 97% sensitivity, however, the amplicon size obtained was 253 bp. Qiu et al. (2009) used subtype specific primers for NA subtyping with 97.3% sensitivity and 91.1%

specificity. The method developed in this study can also amplify NA genes from SIV and human influenza isolates indicating the universal suitability of this method.

Although we did not test this method for amplification of NA gene for the detection of influenza viruses directly from field samples, we believe that this method may be able to do so depending upon concentration of the virus present in the sample.This PCR method for NA subtyping along with HA-subtyping (Hoffmann et al., 2001) can be used for subtyping of influenza A virusesas well as to obtain information on the evolution and any changes (mutations) in the NA gene, which is important to ascertain any changes in antiviral susceptibility of influenza A viruses and emergence of new subtypes.

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Table 1

List of influenza A viruses used in the present study for validation of RT-PCR assay.

Name of the isolate	Subtype	Species	Source
A/TY/KS/4880/80	H1N1	Avian	NVSL
A/CK/NY/3750–7/96	H2N2	Avian	NVSL
A/TY/Ore/71	H7N3	Avian	NVSL
A/TY/ONT/6118/67	H8N4	Avian	NVSL
A/MAL/ALB/650/83	H12N5	Avian	NVSL
A/GULL/MD/704/77	H13N6	Avian	NVSL
A/CK/Germn /49	H10N7	Avian	NVSL
A/DK/Engl/62	H4N8	Avian	NVSL
A/TY/WI/68	H5N9	Avian	NVSL
A/New Jersey/8/76	H1N1	Human	Synbiotics
A/Hong Kong/8/68	H3N2	Human	Synbiotics
A/swine/Kansas/2007	H1N1	Swine	MVDL
A/swine/Missouri/2006	H2N3	Swine	MVDL
A/swine/North Carolina/2007	H3N2	Swine	MVDL

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[b] Synbiotics.

[c] Archives of Minnesota Veterinary Diagnostic Lab., University of Minnesota, St. Paul, MN.

Table 2

List of primers.

N-type	Primer	Sequence (5'–3')	Position (bp)	Accession no.
N1	NA-1.2	F: CAA AGT GTC ATT ACC TAC GAA AAC	159–182	EU980510
		R: TTG TCT GGG CCG GAA ATA CC	607–588	
	NA-1.4	F: TA ATG AGC TGT CCT GTT G	477–498	
		R: ACT GCC TGT TCC ATC ATT G	1010–992	
	NA-1.6	F: GCC AGG CCT CAT ACA AGA TTT TCA	754–777	
		R: CA AGG CCT CAT GCA GTC CA	1271–1249	
	NA-1.8	F: CTG ATG CTA GYG AGG TAA TGT	856–876	
		R: CAA TGG TGA ATG GCA ACT	1410–1393	
N2	NA-2.1	F: AGT GAA AAT GAA TCC AAA TC	1–20	EU980481
		R: GGT CCC CTG CCC AAG TGC	421–404	
	NA-2.3	F: CTG GTG GGG ACA TTT GGG TAA C	336–357	
		R: TAT TCT AGT ATC GGC CTT TCC TG	769–747	
	NA-2.5	F: GG GGA TGA TAG AAA TGC GAC TG	591–614	
		R: AGC CTG AGC GMG AAT CTT TAC TGA	1127–1104	
	NA-2.6	F: GAC AAC TGG AAG GGC TCT AAT A	884–905	
		R: GTG CCA CAA AAT ACG ACA ATA CT	1353–1331	
N3	NA-3.1	F: CTC TGT CAA CGA TAG CCC TTC TCA	53–76	EU980469
		R: CTA TAD GGT GTT CTG TCT TTA	453–474	
	NA3.3	F: GTT AGC TGC GAY AAT GAC AA	370–389	
		R: CTT CCC TCT CGT ATC CA	775–791	
	NA-3.4	F: GAC GGG AAA GAG TGG ATG C	562–580	
		R: GGC CTG GGR GTG TCT GAG T	971–991	
	NA-3.6	F: GAA GAG TGY TCC TGC TAT GT	837–858	
		R: CCA ATT TCC CGA TCC AGG TTC A	1359–1380	
N4	NA-4.1	F: ACC ATC GGC AGT GTT AGT ATT AT	46–68	EF655845
		R: CTG TCC CAT TTG AGT GTT TGT	463–443	
	NA-4.3	F: GGG CAC CAC TGA GCA AGG AC	308–327	
		R: CCA CTY GGG TAA CAG GAA CA	872–849	
	NA-4.5	F: ATG CGA ACA CAA GAG TC	688–705	
		R: CAT CCA TTA GCA TCC CA	1163–11647	
	NA-4.7	F: TTA CCC GAG TGG AAC AGA TA	861–880	
		R: GTC AAA GGG CAA CAG AGC	1422–1405	
N5	NA-5.1	F: GAA TCC AAA TCA GAA AAT AAT AA	11–33	EU743482
		R: TCA ATG CAC GAT AAG GAC	474–456	
	NA-5.3	F: TTT GTC ATA AGA GAA CCA TT	342–365	
		R: ACT TCT CTT TCR TTT GTT ACC ATT	805–782	
	NA-5.5	F: GTG GTG AGA TCA TGG AGA AAG	641–662	

N-type	Primer	Sequence (5’−3’)	Position (bp)	Accession no.
		R: TCG GGA TGA AAT GCT AAC TGT	1118–1098	
	NA-5.7	F: ATG CCA TTG GAG GGA GTG	1015–1032	
		R: TTA TCG ATG TCA AAG GGA AGA	1423–1403	
N6	NA-6.1	F: CAG CAA CAG GAA TGA CAC TAT C	38–59	EF655845
		R: TAA TGC CCT AAA CGG RCT TCT	480–460	
	NA-6.3	F: AAC TAC AAC ACC CAA AAC A	182–205	
		R: GGC CAT CCG TCA TCA CCA C	742–724	
	NA-6.4	F: CAA GCA CCG AGY CCA TAC AA	499–520	
		R: C ATC ACA GTT TCC GCT TCC	1023–1003	
	NA-6.6	F: CCT GTG GTG ATG ACG GAT GG	721–740	
		R: AG CGC TAC TAT ACT ATT GGA TGT	1350–1327	
N7	NA-7.1	F: ATC AAA AAT TAT TCG CAC TC	21–40	EU743316
		R: GAC CAT CCC ACG CAA AG	543–526	
	NA-7.2	F: AAA GTT GAA GGA TGG GTA GTG	278–301	
		R: TAT GTG CCT GGC TGA TCC TTT GAG	832–809	
	NA-7.4	F: A CGC AAG AGT CTG AAT GTG	678–698	
		R: CCT GAG TAT CCT GAC CA	1229–1209	
	NA-7.5	F: AGG ATC AGC CAG GCA CAT AGA	814–837	
		R: GGA AGG AAC CGG ACC CAA CTG	1391–1371	
N8	NA-8.1	F: CCA TTG GGT CAG TAT CCT TAG	34–54	EU743316
		R: CTC CGG TCT TTC ACT GT	463–444	
	NA-8.3	F: TC GAG AGG TCA TGT TTT TGT	326–349	
		R: GTG CTT GTC YGT TTG CTG GTC	756–736	
	NA-8.4	F: CTA ATG GCA CAG TGA AAG ACC	436–456	
		R: CTG GGG AGA CCT GCA CAT AAG TA	970–948	
	NA-8.7	F: GAC AAT TGG ACT GGA AC	877–899	
		R: ATC GAT GTC AAA AGG AAG AAT AGC	1412–1389	
N9	NA-9.1	F: CAC TTC TGC CAC TGC TAT	33–50	EU871838
		R: CGT GTA TTG TTC CAT TTG AGT G	439–460	
	NA-9.3	F: CAG ATG GAG GAG AGG GCA AAT A	232–253	
		R: GAA CAC TAC CGG GCA GAC ACC	715–735	
	NA-9.4	F: GAA TGC ATT GGG TGG TC	532–548	
		R: TTC GGT CGG GGG TTA TCT GT	979–998	
	NA-9.6	F: GGG AAC AGG CAG GGA TTA CTT G	857–878	
		R: ACT TTG TCC TCC TTG GGT CTT CC	1297–1319	
	NA-9.7	F: TAT ATG TAG CCC TGT TCT	960–977	
		R: CAT CAG GCC AGT TCC ATT GTC	1373–1393	

Table 3

Details of NA-subtyping of influenza A viruses isolated from wild birds.

Subtype	Number of isolates	GenBank accession no.
N1	15	CY042614, CY042051, CY042056, CY042072, CY042073, CY042092, CY042093, CY042095, CY042135, CY042151, CY042200, CY042205, CY042209, CY042212, CY042214
N2	14	CY038032, CY038157, CY038162, CY042496, CY042497, CY042584, CY042603, CY042074, CY042075, CY042131, CY042150, CY042163, CY042183, CY042213
N3	4	CY042067, CY042125, CY042143, CY042176
N4	4	CY038262, CY042545, CY042610, CY042026
N5	19	CY038047, CY038057, CY038072, CY038077, CY038082, CY038087, CY038102, CY038112, CY038122, CY038127, CY038132, CY038137, CY038152, CY038232, CY042527, CY042543, CY042557, CY042589, CY042018,
N6	63	CY038037, CY038052, CY038062, CY038067, CY038092, CY038107, CY038117, CY038142, CY038147, CY038182, CY038187, CY038192, CY038197, CY038202, CY038207, CY038212, CY038217, CY038222, CY038227, CY038252, CY038257, CY042420, CY042422, CY042443, CY042451, CY042455, CY042469, CY042507, CY042523, CY042550, CY042558, CY042561, CY042572, CY042599, CY041988, CY041989, CY041990, CY041991, CY041992, CY041993, CY041994, CY041995, CY041998, CY041999, CY042000, CY042001, CY042002, CY042003, CY042005, CY042006, CY042009, CY042010, CY042012, CY042013, CY042017, CY042021, CY042023, CY042027, CY042031, CY042040, CY042041, CY042042, CY042046,
N7	19	CY038042, CY038097, CY038167, CY038177, CY038242, CY042680, CY042019, CY042020, CY042029, CY042032, CY042039, CY042050, CY042057, CY042059, CY042060, CY042066, CY042068, CY042079, CY042084
N8	48	CY038172, CY042411, CY042426, CY042619, CY042623, CY042628, CY042644, CY042649, CY042654, CY042667, CY042676, CY042684, CY042689, CY042694, CY042699, CY042704, CY042709, CY042714, CY041997, CY042004, CY042007, CY042008, CY042011, CY042022, CY042024, CY042028, CY042030, CY042033, CY042035, CY042037, CY042038, CY042043, CY042045, CY042047, CY042049, CY042058, CY042064, CY042070, CY042088, CY042090, CY042115, CY042118, CY042120, CY042122, CY042126, CY042127, CY042134, CY042139
N9	14	CY038237, CY038247, CY042430, CY042594, CY042608, CY042081, CY042082, CY042083, CY042085, CY042089, CY042142, CY042147, CY042181, CY042199