Journal of Clinical Virology 68 (2015) 43–48

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

A simplified Sanger sequencing method for full genome sequencing of multiple subtypes of human influenza A viruses

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A R T I C L E   I N F O

Article history:
Received 13 March 2015
Received in revised form 20 April 2015
Accepted 25 April 2015

Keywords:
Influenza A virus
Sanger sequencing
Full genome sequencing

A B S T R A C T

Background: Full genome sequencing of influenza A viruses (IAV), including those that arise from annual influenza epidemics, is undertaken to determine if reassorting has occurred or if other pathogenic traits are present. Traditionally IAV sequencing has been biased toward the major surface glycoproteins haemagglutinin and neuraminidase, while the internal genes are often ignored. Despite the development of next generation sequencing (NGS), many laboratories are still reliant on conventional Sanger sequencing to sequence IAV.

Objectives: To develop a minimal and robust set of primers for Sanger sequencing of the full genome of IAV currently circulating in humans.

Study design: A set of 13 primer pairs was designed that enabled amplification of the six internal genes of multiple human IAV subtypes including the recent avian influenza A(H7N9) virus from China. Specific primers were designed to amplify the HA and NA genes of each IAV subtype of interest. Each of the primers also incorporated a binding site at its 5′-end for either a forward or reverse M13 primer, such that only two M13 primers were required for all subsequent sequencing reactions.

Results: This minimal set of primers was suitable for sequencing the six internal genes of all currently circulating human seasonal influenza A subtypes as well as the avian A(H7N9) viruses that have infected humans in China.

Conclusions: This streamlined Sanger sequencing protocol could be used to generate full genome sequence data more rapidly and easily than existing influenza genome sequencing protocols.

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1. Background

Influenza viruses belong to the family of Orthomyxoviridae RNA viruses. Influenza A viruses (IAV) contain eight segmented negative strand RNAs of approximately 13.5 kb which encode at least 12 proteins, including polymerase basic protein 2 (PB2) and 1 (PB1), PB1-F2, polymerase acidic protein (PA) and PA-X, hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein 1 and 2 (M1 and M2), and non-structural protein 1 and 2 (NS1 and NS2) [1]. There are 18 types of HA and 11 types of NA [2–5], and the combination of HA and NA subtypes provides the basis for IAV subtype classification (such as H1N1 and H3N2). Most of the subtypes are found in avian, some in swine, human and other species such as bats. While H1N1 and H3N2 are the most common subtypes currently circulating in humans [6], there have also been sporadic cases of human infection with other influenza A subtypes in recent years [7], such as H5N1,H7N9, H10N7, H7N9 and H9N2, H6N1 [8–15].

Both the HA and NA genes of influenza A, particularly those present in the currently circulating human H1N1 and H3N2 viruses continually evolve due to immune pressure from previous infections, leading to antigenic drift in the virus. In contrast, the six internal genes are relatively stable, although genetic reassortment among different virus subtypes can occur within avian and swine hosts, and less frequently in the humans. From time to time, some of these reassortant IAV viruses from animal sources may develop an ability to infect humans, such as was seen with the 2009 pandemic H1N1 virus, which appears to have been a swine reassortant virus [16]. Full genome sequencing of IAV is the standard way to identify the origin of a newly reassortant virus and it is also a powerful tool to help elucidate the molecular characteristics of the virus such

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http://dx.doi.org/10.1016/j.jcv.2015.04.019
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as host range, replication efficiency, transmissibility and virulence [17–20].

There are two main technologies that are currently in use for influenza full genome sequencing, namely the next generation sequencing (NGS) and traditional Sanger sequencing. The entire genome sequences regardless of the subtypes can be amplified using universal IAV primers, and then sequenced using NGS [21–23], however, the cost and availability of NGS equipment, coupled with the need for complicated bioinformatics analysis, are major barriers for many laboratories to introduce this new technology. Therefore, Sanger sequencing is still widely used in most of the laboratories involved in influenza surveillance and research.

2. Objectives

To develop a simple Sanger sequencing method that is able to amplify and sequence the full genomes of multiple circulating seasonal human IAV subtypes using the minimum number of primers.

3. Study design

3.1. Primer design

Sequence data for all eight genes of A(H3N2), A(H1N1), A(H1N1pdm09) were downloaded from the global initiative on sharing avian influenza data (GISAID) website, alignment of individual genes was done in Geneious 5.1 (Biomatters Ltd-New Zealand). HA and NA primers were designed specific for each IAV subtype unless otherwise stated. Primers for the internal gene segments were designed at a minimum to cover all known IVAs belonging to the seasonal H1N1, H3N2 and H1N1pdm09. All genes except the Matrix gene and NS gene (where only single sets of primers were used) were amplified with 2–3 overlapping PCR segments to ensure that good quality sequence data could be obtained from both directions in one segment, all primers were each tagged with either M13F or M13R universal sequencing primer at the 5′ end, so that only two primers (M13F and M13R) were needed for sequencing all the PCR products (Fig. 1). Altogether, 17 pairs of primers were needed to amplify the whole influenza A genome of each subtype of IAVs, of which 13 pairs were universal primers that could amplify the six internal gene segments of all known IAVs currently circulating in humans (Table 1). Some of the primers were used were those recommended by the US CDC (Atlanta, Georgia) protocol as indicated in Table 1.

3.2. Virus samples

Samples were received at the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne as either IAV isolates in Madin–Darby canine kidney (MDCK ATCC CCL-34) cells or original respiratory clinical specimens (e.g. nasal swabs, throat swabs, nasal aspirates, nasopharyngeal swabs) from WHO National Influenza Centres, and other regional laboratories and hospitals in Australia, New Zealand, and the Asia/Pacific regions. If required virus samples were cultured in MDCK cells or embryonated hens eggs as previously described [24,25].

3.3. RNA extraction and RT-PCR

RNA was extracted from 140 μl of a virus culture or an original specimen using

QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instruction.

cDNA was made from each RNA sample using ThermoScript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen), with Uni12 primer [26], then 2 μl of the cDNA was amplified in a 50 μl volume using the Platinum Taq DNA High Fidelity Polymerase kit (Invitrogen) containing specific gene primers as shown in Table 1 according to the manufacturer's instructions.

3.4. Sequencing and sequence analysis

RT-PCR products were purified by ExoSAP-IT (GE Healthcare), and then subjected to sequencing reactions using either M13F or M13R primer with Big Dye Terminator Reaction Mix (Applied Biosystems). The reaction products were purified by Big Dye XTerminator Purification Kit (Applied Biosystems) and run on ABI 3500 XL sequencer. Sequencing results were analysed using the DNASTAR Lasergene 9 package.

4. Results

4.1. Validation of IAV internal primers

A total of 13 pairs of primers were designed for the six internal gene segments (PB1, PB2, PA, NP, M, NS) of IAV as described in Methods and Materials. These primers were tested on 25 seasonal H1N1, 120H3N2, 130H1N1pdm09 and 10 influenza B MDCK grown viruses, with sample dates from 1980 to 2014. The represented viruses sequenced are listed in Table 2. When tested on these viruses the primers were able to produce distinct PCR products that could be easily sequenced, and they also performed at a similar efficiency when tested on the different influenza A subtypes tested (H1N1, H1N1pdm09 and H3N2), with no cross reactivity against influenza B viruses. The analytical sensitivity was also determined on cDNA derived from serially diluted RNA samples, and similar levels in the limit of detection (LOD) were found for respective gene segments of various subtypes, ranging from 50 to 50,000 copies/reaction (data not shown). The LOD for NS and NP genes were the most sensitive (50 copies/reaction), whereas some polymerase fragments had a lower sensitivity (50,000 copies/reaction).

4.2. Specificity and sensitivity of HA and NA primers

The subtype specific HA and NA primers were tested for specificity on over 200 seasonal H1N1 viruses (sample dates from 1934 to 2009), 1500H3N2 viruses (sample dates 1976–2014) and 1500H1N1pdm09 viruses (sample dates 2009–2014). All the primers tested were specific for the HA and NA genes of their corresponding subtypes, with no cross-reaction to the other IAV subtypes or influenza B virus. The LOD on HA and NA for all subtypes were found in the range of 500–5000 copies/reaction.

4.3. Evaluation of IAV internal gene primers for amplification of other subtypes

All the internal primers were further tested on a number of influenza A subtypes discovered either in humans or animals. Results revealed that they were able to amplify the internal genes of all selected influenza A viruses including H2N2 (A/Singapore/1/1957), H10N7 (A/Chicken/Queensland/1/2012), H1N2 (A/Brisbane/116/2002 and A/Sydney/12/2003), and the avian H7N9 virus (A/Anhui/1/2013) (Table 2). When these primers were tested on selected H9N2 and H5N1 viruses, only the whole NP, NS and MP genes, but not the three polymerase genes, could be amplified successfully. Analysis of the polymerase genes of these subtypes revealed a high degree of diversity, making it difficult to design primers that also covered both H5N1 and H9N2 polymerase genes.

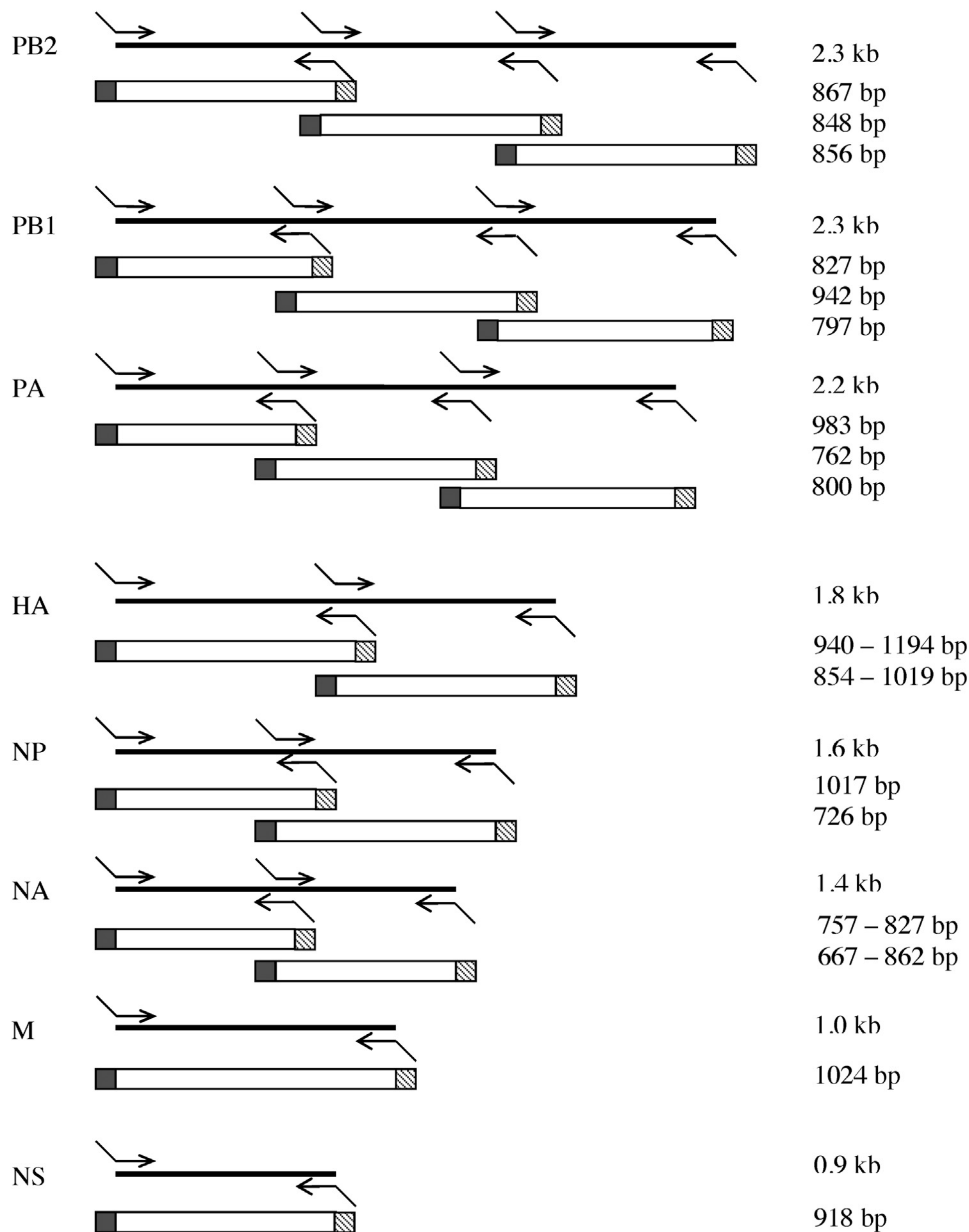

Fig. 1. RT-PCR scheme illustrating all 8 segments of FluA amplification using M13 tagged primers. All 6 internal primers are universal for all subtypes of seasonal human IAV, the HA and NA primers are subtype specific. All of the forward primers are tagged with M13F universal sequencing primer at the 5′ end ( ), all the reverse primers with M13R universal sequencing primer at the 5′ end ( ) to facilitate downstream sequencing.

4.4. Sequencing and sequence analysis of PCR products

All the PCR products generated from various subtypes were sequenced using M13F and M13R primers. High quality sequence data were generated which was reflected by a long contiguous read length (CRL) (up to 900 bp), and a high Phred quality value (QV20+ for over 99% of the bases). The sequence data derived from both ends using the two M13 primers effectively covered the whole fragment for all the PCR products, alleviating the need for further gap sequencing using additional internal primers. The assembled sequences were blasted against the sequence data available in GenBank. Results revealed that all the sequenced IAVs belonged to their corresponding genes/subtypes and exhibited a high degree of homologies to the viruses sequenced from around the same time period.

4.5. Sequencing of IAV from original specimens

Further tests were performed to determine whether this set of PCR primers could efficiently amplify IAV genes from original specimens including nasal swabs and nasopharyngeal aspirates collected from patients. A total of 222 original specimens confirmed previously by real-time RT-PCR to carry various subtypes of IAVs were used for HA/NA PCR and sequencing. Among them, 12 contained the seasonal H1N1, 120H1N1pdm09, and 90H3N2 viruses, with a real-time Ct value on IAV matrix gene ranging from 16 to 33. Full HA and NA genes were successfully sequenced from more than 90% of the samples. Most of the samples that could not be amplified for one or more fragments of HA and/or NA had a high Ct value (>33), except for a few with a lower Ct value which most likely contained degraded RNA. Twelve of the original specimens

Table 1
PCR primers for full genome sequencing of human IAV.

Segment	Primer name	Primer sequence (5'–3')	Nucleotide position[a]	Expected amplicon size (bp)[b]	Source
PB2 Fragment 1	A-PB2-I-M13F	TGTAAAACGACGGCCAGTCTCGAGCAAAAGCAGGTCAA	(−31)– (−20)	903	This study
	A-PB2-I-M13R	CAGGAAACAGCTATGACCGATGCTARTGGRTCTGCTG	836–813		This study
PB2 Fragment 2	A-PB2-II-M13F	TGTAAAACGACGGCCAGTGGGARCARATGTACACTC	719–736	884	This study
	A-PB2-II-M13R	CAGGAAACAGCTATGACCYTGYGTTTCACTGAC	1566–1552		This study
PB2 Fragment 3	A-PB2-III-M13F	TGTAAAACGACGGCCAGTGCAAAATGGGWGTRGATG	1442–1459	892	This study
	A-PB2-III-M13R	CAGGAAACAGCTATGACCTAGAAACAAGGTCGTT	2341–2297		This study
PB1 Fragment 1	A-PB1-I M13F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGCAAACCAT	(−24)–(−5)	863	This study
	A-PBI-I-M13R	CAGGAAACAGCTATGACCTGTTCAAGCTTTTCRCAWATGC	803–782		This study
PB1 Fragment 2	A-PB1-II-M13F	TGTAAAACGACGGCCAGTCRATGACCAAAGATGCWGA	677–695	978	This study
	A-PB1-II-M13R	CAGGAAACAGCTATGACCAAGGTCATTGTTTATCATRTTG	1618–1597		This study
PB1 Fragment 3	A-PB1-III-M13F	TGTAAAACGACGGCCAGTGTGGCYAATTTYAGCATGGAG	1504–1524	833	This study
	A-PB1-III-M13F	CAGGAAACAGCTATGACCAGTAGAAACAAGGCATTT	2317–2300		This study
PA Fragment 1	A-PA-I-M13F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGTACTGAT	(−24)–(−6)	1019	This study
	A-PA-I-M13R	CAGGAAACAGCTATGACCGGYTCTTTCCAKCCAAAG	959–942		This study
PA Fragment 2	A-PA-II-M13F	TGTAAAACGACGGCCAGTCMAARTTCCTSCTGATG	839–855	798	This study
	A-PA-II-M13R	CAGGAAACAGCTATGACCTCMAGTCTYGGGTCAGTGAG	1600–1582		This study
PA Fragment 3	A-PA-III-M13F	TGTAAAACGACGGCCAGTGCATCCTGTGCAGCMATGGA	1414–1433	836	This study
	A-PA-III-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGTACCTTTT	2213–2193		This study
NP Fragment 1	A-NP-I-M13F	TGTAAAACGACGGCCAGTCAGGGTWRATAATCACTCAMTG	(−37)–(−16)	1053	This study
	A-NP-I-M13R	CAGGAAACAGCTATGACCTGRCTCTTGTGWGCTGG	980–964		This study
NP Fragment 2	A-NP-II-M13F	TGTAAAACGACGGCCAGTCTGAGRGGRTCAGTTGC	795–811	762	This study
	A-NP-II-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTATTTTTC	1520–1499		This study
M	A-MP-M13F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGTAG	(−25)–(−10)	1060	US CDC
	A-MP-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGTAGT	999–986		US CDC
NS	A-NS-M13F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGGTGACAAAGACA	(−27)–(−3)	954	This study
	A-NS-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTTTTTAT	891–868		This study
sesonal H1N1					
HA Fragment 1	H1-I-M13F	TGTAAAACGACGGCCAGTCAACCAAAATGAAAG	(−8)–7	1098	US CDC
	H1-I-M13R	CAGGAAACAGCTATGACCGCAATGGCTCCAAACAAACCTCT	1054–1032		US CDC
Fragment 2	H1-II-M13F	TGTAAAACGACGGCCAGTAGAATYAAYTACTACTGGAC	727–746	1055	US CDC
	H1-II-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTTTTYCTYATATT	1745–1716		This study
NA Fragment 1	uNA-I-M13F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGAGT	(−20)–(−6)	793	US CDC
	N1-I-M13R	CAGGAAACAGCTATGACCGGRCCATCGGTCATTATG	720–737		US CDC
Fragment 2	N1-II-M13F	TGTAAAACGACGGCCAGTGGAATTTCYGGTCCAG	580–595	898	US CDC
	uNA-II-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGAG	1441–1427		US CDC
pdm H1N1					
HA Fragment 1	pdmHA-I-MI3F	TGTAAAACGACGGCCAGTATGAAGGCAATACTAGTAG	1–19	976	This study
	pdmHA-I-MI3R	CAGGAAACAGCTATGACCGATCGGATGTATATTCTGAAATGG	940–922		This study
Fragment 2	pdmHA-II-MI3F	TGTAAAACGACGGCCAGTGATTGCAATACAACTTGTC	871–889	890	This study
	pdmHA-II-MI3R	CAGGAAACAGCTATGACCTCATGCTTCTGA	1724–1712		This study
NA Fragment 1	pdmNA-I-MI3F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGAG	(−20)–(−7)	863	This study
	pdmNA-I-MI3R	CAGGAAACAGCTATGACCGTGATAATTAGGGGCATTC	823–807		This study
Fragment 2	pdmNA-II-MI3F	TGTAAAACGACGGCCAGTGACAGGCCTCATACAAGATCTTC	746–768	703	This study
	pdmNA-II-MI3R	CAGGAAACAGCTATGACCAATTACTTGTCAATGG	1412–1397		This study
H3N2					
HA Fragment 1	H3-I-M13F	TGTAAAACGACGGCCAGTAAAGCAGGGGATAATTCTA	(−25)–(−7)	1230	This study
	H3-I-M13R	CAGGAAACAGCTATGACCATTGCTGCTTGAGTGCTT	1169–1152		This study
Fragment 2	H3-II-M13F	TGTAAAACGACGGCCAGTGGTTACTTCAAAATAC	814–829	937	This study
	H3-II-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTTT	1733–1714		This study
NA Fragment 1	uNA-I-M13F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGAGT	(−19)–(−5)	878	US CDC
	N2-I-M13R	CAGGAAACAGCTATGACCCGACATGCTGAGCACTYCCTGAC	823–804		This study
Fragment 2	N2-II-M13F	TGTAAAACGACGGCCAGTGAACTTGTRCAGTRGTAATG	704–723	780	US CDC
	uNA-II-M13R	CAGGAAACAGCTATGACCAGTAGAAACAAGGAG	1447–1433		US CDC

[a] The start codon "ATG" of each gene is designated position #4′5#, some primers start before the start codon, and are given a negative number.
[b] The amplicon sizes include the M13F and M13R primer lengths.

(two containing H3N2 and 10H1N1pdm09) were chosen for full genome sequencing. All 12 samples had a Ct value on IAV matrix gene between 17 and 27. Results revealed that sequencing the full length of all eight genes was achieved with all of the 12 original samples.

5. Discussion

Full genome sequencing of IAV has been increasingly used for research and global surveillance of influenza viruses. Traditionally, this has been done by Sanger sequencing; however, the most critical determinant for a successful full genome Sanger sequencing strategy of IAV is the availability of primers that are able to target only the IAV sequences reliably but not the sequences of host genes or other pathogens. Due to the high diversity of IAVs, most of the Sanger-based methods developed to date are IAV subtype specific and often require the use of large numbers of primers. For example, the method reported by Lee et al [27] is applicable only to H3N2 subtype viruses, and it involves the use of 19 pairs of PCR primers for full genome amplification of the viruses, followed by an

Table 2
Representatives of viruses from different subtypes of IAV being sequenced using the IAV internal primers.

Subtype	Virus designation	Year	Isolate type	Virus host	GISAID Isolate ID	Genes sequenced
H3N2	A/Victoria/186/1982	1982	Egg	Human	EPI_ISL_173279	PB2,PB1,PA,NP,M,NS
	A/Sydney/2/1993	1993	MDCK	Human	EPI_ISL_173280	PB2,PB1,PA,NP,M,NS
	A/Nauru/5/2011	2011	Clinical specimen	Human	EPI_ISL_95078	PB2,PB1,PA,NP,M,NS
	A/South Australia/313/2011	2011	Clinical specimen	Human	EPI_ISL_118601	PB2,PB1,PA,NP,M,NS
	A/Newcastle/1/2013	2013	MDCK	Human	EPI_ISL_141550	PB2,PB1,PA,NP,M,NS
sH1N1	A/Queensland/1/1983	1983	Egg	Human	EPI_ISL_173281	PB2,PB1,PA,NP,M,NS
	A/Victoria/4/1986	1986	Egg	Human	EPI_ISL_173282	PB2,PB1,PA,NP,M,NS
	A/Victoria/1/1991	1991	Egg	Human	EPI_ISL_173276	PB2,PB1,PA,NP,M,NS
	A/Perth/5/1995	1995	MDCK	Human	EPI_ISL_173283	PB2,PB1,PA,NP,M,NS
H1N1pdm09	A/Auckland/1/2009	2009	Clinical specimen	Human	EPI_ISL_30628	PB2,PB1,PA,NP,M,NS
	A/Guam/2/2010	2010	Clinical specimen	Human	EPI_ISL_76617	PB2,PB1,PA,NP,M,NS
	A/Townsville/5/2013	2013	MDCK	Human	EPI_ISL_148750	PB2,PB1,PA,NP,M,NS
H2N2	A/Singapore/1/1957	1957	Egg	Human	EPI_ISL_130414[a]	PB2,PB1,PA,NP,M,NS
H1N2	A/Brisbane/116/2002	2002	MDCK	Human	EPI_ISL_173277	PB2,PB1,PA,NP,M,NS
	A/Sydney/12/2003	2003	MDCK	Human	EPI_ISL_173278	PB2,PB1,PA,NP,M,NS
H10N7	A/Chicken/Queensland/1/2012	2012	Egg	Chicken	EPI_ISL_129740	PB2,PB1,PA,NP,M,NS
H7N9	A/Anhui/1/2013	2013	Egg	Human	EPI_ISL_138739[a]	PB2,PB1,PA,NP,M,NS
H9N2	A/Hong Kong/33982/2009	2009	MDCK	Human	EPI_ISL_69733[a]	NP,M,NS,
H5N1	A/Turkey/15/2006	2006	MDCK	Human	EPI_ISL_12586[a]	NP,M,NS,

[a] Our sequences match the sequences previously existed in the database, with the accession numbers listed.

additional 40 primers for sequencing the PCR products. Similarly, 198 primer sets were used initially to amplify and sequence the full genome of the first novel avian A(H7N9) virus that arose in China in 2013 which has now resulted in hundreds of human infections and many deaths [10].

In this study, we demonstrated that using a set of only 13 pairs of primers we successfully amplified and sequenced all of the six internal gene segments from multiple IAV subtypes. Although these primers were mainly designed to sequence the circulating human IAV subtypes, they could also be used to sequence a limited number of other IAV subtypes from human and animal sources. For example, this set of primers successfully amplified and sequenced the full internal genes from H2N2, H10N7 and H1N2 viruses as well as the recently emerged avian H7N9 virus from China. Due to the high diversity of other IAVs, however, we found it very difficult, if not impossible to design a set of primers that could universally amplify the six internal genes from all influenza A subtypes.

In contrast to the six internal genes, the genes for HA and NA are notorious for their high heterogeneity which makes it impossible to design a set of primers that are applicable to the HA and NA genes of all subtypes. Therefore, in this study, the HA and NA genes of only three IAV subtypes were chosen as the targets for full genome sequencing because of their presence in the general human population in recent years. The primers designed were evaluated extensively for their subtype specificities on over 3200 cultured IAVs of different subtypes including H1N1, H3N2 and H1N1pdm09 viruses. The results demonstrated that the HA- and NA- specific primers could be applied to a wide range of viruses isolated from 1934 to the present time, and that no cross-reactivity between different IAV subtypes or to influenza B virus was detected with any of the primers tested. Most importantly, all the primers reported in this study did not cross react with other pathogens or host genes. In addition, the use of a single pair of M13 primers for all sequencing reactions not only simplifies the sequencing procedure but also ensures the generation of high quality sequences consistently.

With our simple and streamlined full genome Sanger sequencing protocol, up to five viruses can be sequenced overnight with just two 96-well sequencing plates on ABI 3500XL sequencer. The sequence data can be analysed using many standard programs quickly, leading to a much shorter turnaround times than are usually seen with NGS, especially in the case when only a small number of viruses are sequenced. Given the sensitivity of the primer pairs these assays could be used on both cell culture-derived and egg-grown virus isolates, as well as on clinical respiratory specimens when there is sufficient virus present. In conclusion, this simple Sanger sequencing protocol provides a useful backup sequencing strategy in this current era of NGS, and also enables smaller laboratories that do not have NGS equipment to use this Sanger sequencing method to rapidly and reliably sequence the circulating human IAV genomes from their regions.

Conflict of interest

I approve the final manuscript.

Funding

The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

Ethical approval

Not required.

Acknowledgements

The authors would like to thank all laboratories that supplied samples used in these studies including the WHO National Influenza Centres, WHO Influenza Collaborating Centres, and other regional laboratories and hospitals in Australia, New Zealand, and the Asia/Pacific region. The authors gratefully acknowledge John Barnes and Catherine Smith for sharing their US CDC primer sequences.

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