

Influenza A Virus: Phylogeny of Neuraminidase Primers and Amplification of Polymerase Basic Protein 2 and Neuraminidase Genes

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Influenza A virus is a highly contagious agent that causes bird flu. To date, 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes are identified antigenically and can form any combinations or mutations with each other to confer non or low pathogenicity to high pathogenic strains. Mutations in viral segments that are derived from avian isolates represent a novel subtypes to which human population is infected by influenza pandemics. In this work, polymerase basic protein 2 (PB2) gene segment of 8 different avian influenza subtypes were cloned to obtain more DNA samples for future work such as PB2 sequencing and to test HA primer annealing with PB2 gene. PCR amplification of NA gene segment of 3 different avian influenza subtypes was the second aim of this work to test primer universal for NA genes. Determination of the aligned sequences between 9 NA subtypes and NA primer PCR products was the second aim of this work, based on BLAST result homology 100% and phylogenetic trees of clustal

Keywords: Influenza A virus, phylogeny, amplification, PB2 gene, NA gene

Avian influenza pathogenic virus is a member of the family Orthomyxoviridae (Lamb *et al.* 1996; Lamb *et al.* 2007), divided into subtypes on the basis of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) (Hoffmann *et al.* 2000; Wright *et al.* 2001). Studies of pathogenicity showed that the optimal combination of HA and NA enabling the cleavage of HA was important. A balance in HA and NA activities is crucial; there must be enough HA activity to facilitate virus binding and enough NA activity to allow release of virus progeny (Mitnaul *et al.* 2000; Hulse *et al.* 2004). Therefore, HA and NA detection of influenza A virus have been done effectively using polymerase chain reaction (PCR) strategy, HA and NA universal primers to obtain information that may be related with the detection of viral infection, pathogenicity and host range of influenza A virus. In addition, phylogenetic lineages and BLAST homology results showed 100% between 9 NA viruses and NA primer PCR products which are essential to develop alignment and shared homology between subtypes.

The accumulation of mutations in viral segments such as polymerase basic protein 2 (PB2), M2 and NA genes can generate new chances to increase viral ability to infect species outside of natural reservoir and produce pathogenically various subtypes of virus. Therefore, it is important to identify these mutations to detect this viral infection and pathogenicity. For example, the mutation Ser64Ala in M2 protein gives rise to amantadine resistance (Barr 2005; Komadina *et al.* 2005; Russel *et al.* 2008). A glutamic acid to lysine substitution at position 627 in the PB2 protein (Hatta *et al.* 2001; Watanabe *et al.* 2008) has been associated with increased virulence of H5N1 viruses (Vesudevan *et al.* 2005; Alvarez *et al.* 2008). Moreover, Katz *et al.* (2000) analyzed that residues Thr-223 in the NA gene was unique to the H5N1 viruses of low pathogenicity and that Glu-355 in PB2 was found only in the human H5N1 viruses of low pathogenicity isolated from birds in Hong Kong in 1997. Mutations are also

associated with the antiviral drug resistance, Oseltamivir, such as the H5N1 strains with the H274T, R292K and N294S mutations (Moscona 2005; Moscona 2008; Peters *et al.* 2008).

MATERIALS AND METHODS

DNA Cloning of PB2 Gene Avian Influenza A Virus Subtypes: Preparation of *Escherichia coli* Strain DH5 α Competent Cells According to the Protocol Provided by Nishimura *et al.* (1990). *Escherichia coli* strain DH5 α was used as host cells for amplification of plasmid DNA. Bacteria cultures were diluted 1:100 in fresh LB broth with ampicillin and then incubated further at 37 °C in a shaking incubator for overnight. 0.5 mL overnight culture was transferred into 50 mL solution A (LB, MgSO₄H₂O, glucose) in the flask at room temperature. The cultures were then incubated at 37 °C with continuous shaking for about 3-4 h to reach a cell concentration of 8-12 x 10⁸ cells mL⁻¹ mid-log suspension culture indicating an OD₆₀₀ of 0.8-1.2. This culture was concluded when an optical density measured at 600 nm (OD₆₀₀) is 1.3. The culture was then transferred to microcentrifuge tubes and pelleted at 1500 g for 10 min at 4 °C. The supernatant was discarded while the pellet was resuspended in 0.5 mL solution A and 2.5 mL solution B (glycerin, PEG, MgSO₄H₂O) and aliquoted in 0.1 mL each in 1.5 mL microcentrifuge tubes. These aliquots were then stored at -80 °C.

Ligation. A total volume of 10 μ L ligation reaction consisted of 5 μ L rapid ligation buffer 2x, 1 μ L *Taq* DNA polymerase (Promega), and an appropriate volume of insert DNA such that pGEM-T essay vector: insert DNA ratio was approximately 1:2. Reactions were made up to final volumes using sterile distilled water and incubated at room temperature for an hour and then stored at 4 °C until transformation was carried out.

Heat Shock Transformation. For transformation, 100 μ L competent *E. coli* strain DH5 α cells were mixed with 10 μ L A-tailed ligation product (PB2 gene of H1N9 or H3N8 in p-GEM-T easy vector) and chilled in ice for 10 min prior to heat shock treatment in a 42 °C water bath for 1 min. The cells

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were immediately placed back in ice for another 20 min. The reaction mixture was then incubated in 1 mL prewarm LB at 37 °C for 1 h in a falcon tube. 100 µL bacterial suspension was spread per preheated 100 µg mL⁻¹ of ampicillin selective LB agar plates that were added with 100 µL IPTG and 20 µL X-gal for overnight incubation at 37 °C. The remaining bacterial suspension was also spread per LB agar plates and incubated overnight at 37 °C.

Screening of Positive Colonies. p-GEM-T easy plasmid (Promega) was the vector used for all cloning work. Putative transformants containing this vector with the desired insert were identified based on blue/white screening. Putative positive colonies were identified as white colonies and approximately 10 white colonies per plate were isolated using sterile pipette tip per colony. We did not find any white colonies that grew on agar plates because the ligation and transformation of incompetent *E. coli* DH5α cells were failed. Consequently, we repeated the ligation and transformation with another type of competent cells such as *E. coli* Top10 (Invitrogen) cells.

Ligation. A total volume of 10 µL ligation reaction consisted of 5 µL rapid ligation buffer 2x, 1 µL *Taq* DNA polymerase (Promega), and an appropriate volume of insert DNA such that pGEM-T assay vector: insert DNA ratio was approximately 1:2. Reactions were made up to final volumes using sterile distilled water and incubated at 4 °C for overnight and then, transformation was carried out after successful complementary annealing of vector 3'- thymidine (T)-overhangs with deoxyadenosine (A)-tailed PCR inserts enables ligation.

Rapid Chemical Transformation. For transformation, 50 µL competent *E. coli* Top10 (Invitrogen) cells were mixed with 10 µL each A-tailed ligation product (PB2 gene of H5N3, H6N5, H7N7, H11N6, H12N9, or H15N9 shown in table 1, in p-GEM-T easy vector) and chilled in ice for 5 min. 10 µL of cells was spread on a pre-warm LB agar plate containing 100 µg mL⁻¹ ampicillin, 100 µL IPTG and 20 µL X-gal. The remaining (50 µL) of cells was also spread on another LB agar plate. These agar plates were incubated overnight at 37 °C.

Screening of Positive Colonies. p-GEM-T easy plasmid (Promega) was the vector used for all cloning work. Putative transformants containing this vector with the desired insert were identified based on blue/white screening. Putative positive colonies were identified as white colonies and approximately 10 white colonies per plate were isolated using sterile pipette tip per colony. Each tip was immersed in 5 mL falcon tubes containing 2 mL LB broth supplemented with 100 µg mL⁻¹ ampicillin. The bacterial suspension was grown overnight in a 37 °C shaking incubator. Positive colonies are determined with plasmid purification followed by nanodrops

spectrophotometer, restriction digestion and these clones are cryopreserved at -20 °C until use.

Plasmid Purification. The alkaline method was used for purification of plasmid DNA using the QIAprep Spin MiniPrep Kit (Qiagen). Briefly, putative transformants (white colonies) were cultured in 2 mL LB broth supplemented with 100 µg mL⁻¹ ampicillin for overnight. Bacterial culture grew at 37 °C in a shaking incubator. Plasmid extraction was carried out in accordance with the manufacturer's recommendation. The eluted DNA is stored in -20 °C freezer.

Determination of DNA Concentration. The absorbance of DNA was measured at 260 nm (A_{260}) using a Nanodrop Ultraviolet/Visible Spectrophotometer. DNA concentrations were estimated with the assumption that 50 µg mL⁻¹ double stranded DNA has an absorbance at A_{260} of 1. The Nanodrop spectrophotometer was utilized in all absorbance and DNA/RNA concentration determination. 2 µL each sample of eluted DNA was dropped and measured at $\mu = 260$, DNA -50 using Spectrophotometer program of ND1000 V3.1.2, nucleic acid. Eluted DNA was pure if 260/280 is 1.8-2.0. The concentration of eluted DNA was also measured to obtain information in DNA dilution.

Restriction Endonuclease Digestion of plasmid DNA. Restriction endonuclease digests were performed with a 20 µL digestion reaction mix consisting 2 µL H buffer 10x (Roche) and an appropriate amount of DNA such as restriction enzyme: plasmid DNA ratio was approximately 1:10. Reactions were made up to final volumes using sterile distilled water. The enzyme *EcoRI* was used in all restriction digests and thus, the reaction mix was carried out using the PCR cycling program (37 °C for 90 min, 65 °C for 15 min and 4 °C until end). Digested products were visualized on 2% agarose gels.

Agarose Gel Electrophoresis of DNA. Agarose gel electrophoresis of DNA was typically run on a 1.5-2% (w/v) agarose gel stained with ethidium bromide in 1x TAE buffer. DNA samples were combined with 6x loading dye. The size of DNA fragments were determined by comparison to a standard 1 kb DNA Ladder Mix or 2 kb DNA Hyperladder II, visualized by ultra violet illumination.

Polymerase Chain Reaction Amplification of NA Genes. PCR involves repetitive thermal cycling composed of three steps: i, melting temperature (94 °C) to denature DNA; ii, annealing to cool the mixture (step down temperature from 56-42 °C) allowing NA primers to anneal target DNA sequences; and iii, extension to elongate DNA with dNTP by polymerase. PCR amplification of 3 subtypes of Avian NA genes of Influenza virus subtypes (H6N5, H7N7 and H12N9) were essential as a separate step following cDNA composition via RT-PCR due to limited starting volumes of RNA. Amplification was carried out in a 50 µL reaction consisting of 5 U µL⁻¹ *Taq* DNA polymerase, 10x *Taq* reaction buffer, 50 mM MgCl₂, 10 mM dNTP mix, 100 µM of each forward and reverse NA primer, an appropriate volume of distilled water and template DNA as optimized from previously obtained RT cDNA. PCR reaction was carried out using the MasterCycler PCR® Machine (Eppendorf, QLD). PCR products were stored at -20 °C (freezer) and were also visualized on 1.5% agarose gels.

Table 1 Polimerase basic protein 2 (PB2) genes of Influenza A Virus Subtypes

Subtype	Avian species	Reference
H1N9	A/Shelduck/WA/1757/78	8705-22-1100
H3N8	A/Avian/669/WA/78	8510-15-1215
H5N3	A/Shearwater/Aust/75	8508-16-1600
H6N5	A/Shearwater/Aust/72	8507-10-1200
H7N7	A/Chicken/Victoria/1/85	8507-31-1430
H11N6	A/Tern/Aust/75	8610-15-1330
H12N9	A/RNStint/WA/5754/84	9010-05-1330
H15N9	A/Shelduck/WA/1762/79	9008-18-1000

Extraction of DNA Fragments from Agarose Gels. The MinElute Gel Extraction Kit Protocol using a microcentrifuge (Qiagen) was utilized for the isolation of specific DNA fragments from agarose gels. Experimentation was performed according the manufacturer's instructions. Generally, gel extractions were performed to elute DNA from gels contained desired DNA bands of PCR products with an appropriate amount of gel such that gel: buffer QG ratio was approximately 1:3 and gel: isopropanol was approximately 1:1, and added another buffer (PE) and distilled water. To elute more DNA, second clean up was carried out by adding distilled water to the spin column. Eluted DNA was stored at -20 °C.

Blast Searches and Phylogenetic Tree Development and Basic Local Alignment Search Tool (BLAST) Searches. Sequence data was compared to GenBank DNA Database using BLASTn searches to determine the alignment (% identity) between NA primers (table 2) and all virus origin (virus nucleotide database) using the National Centre for Biotechnology Information (NCBI) BLAST network server available from <http://www.ncbi.nlm.nih.gov/>

Phylogenetic Tree Development. Phylogenetic trees were developed to test variability for regions of sequence bounded by PCR primers. 3 pairs of NA primers (5F and 10R, 8F and 10R, 10F and 11R) were aligned against a selection of genbank database sequences using ClustalX v 1.83 available from <http://bips.u-strasbg.fr/Documentation/ClustalX>. Sequences based on Influenza Sequence Database-<http://www.flu.lanl.gov/> and NA primer sequences were uploaded as FASTA files and a preliminary complete alignment was done. Aligned sequences were viewed, and then sequences were cropped to do alignment on sequence range defined by primers. Cropped sequences were realigned and saved as a new FASTA files. A region sequences defined by primers was aligned to draw bootstrapped NJ trees and to open the trees using Treeview. 1000 bootstrap values were carried out and can be shown at the nodes of the trees shown in Fig 1, 2, and 3.

Mutations in NA Primer PCR Products. Mutations in the NA primer products were specifically targeted for research in this project. Mutations positions of the NA primers have been reported to increase infection, pathogenicity and resistance to antiviral drugs. Thereby, it is essential to determine any mutations that occur clinically in NA primer PCR products.

RESULTS

Amplification of PB2 Gene Segment DNA via Cloning. DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length of 986 bp. Lane 1 consisted of 2 kb DNA Hyperladder II. Lane 2 consisted of positive control, contained plasmid

DNA without EcoRI enzyme. Lane 3 showed negative control, contained EcoRI enzyme without plasmid DNA. Lane 4-13 each denoted p-GEM-T easy vector DNA length product of 3015 bp and H5N3/A/Shearwater/Aust/75 PB2 full length product of 986 bp, clones 1-10.

DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length which was expected to be 986 bp. Lane 1 consists of 2 kb DNA Hyperladder II. Lane 2 shows negative control, contains EcoRI enzyme without plasmid DNA. Lane 3-12 each denotes p-GEM-T easy vector DNA length product of 3015 bp and H6N5/A/Shearwater/Aust/72 PB2 full length product of 986 bp, clones 1-10.

DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length of 986 bp. Lane 1 consists of 2 kb DNA Hyperladder II. Lane 2 shows negative control, contains EcoRI enzyme without plasmid DNA. Lane 3-12 each denotes p-GEM-T easy vector DNA length product of 3015 bp and H11N6/A/Tern/Aust/75 PB2 full length product of 986 bp, clones 1-10.

DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length which was expected to be 986 bp. Lane 1 consists of 2 kb DNA Hyperladder II. Lane 2 consisted of positive control, contained plasmid DNA without EcoRI enzyme. Lane 3 showed negative control, contained EcoRI enzyme without plasmid DNA. Lane 4-13 each denoted p-GEM-T easy vector DNA length product of 3015 bp and H15N9/A/Shelduck/WA/1762/79 PB2 full length product of 986 bp, clones 1-10.

DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length of 986 bp. Lane 1 consisted of 2 kb DNA Hyperladder II. Lane 2 showed negative control, contained EcoRI enzyme without plasmid DNA. Lane 3 and 7 each consisted of positive control, contained plasmid DNA without EcoRI enzyme. Lane 4-5 each denoted p-GEM-T easy vector DNA length product of 3015 bp and H7N7/A/Chicken/Victoria/1/85 PB2 full length product of 986 bp, clones 1-2. Lane 6 showed only p-GEM-T easy vector DNA length product of 3015 bp, without any insert DNA. Lane 8-10 depicted p-GEM-T easy vector DNA length product of 3015 bp and H12N9/A/RNstint/WA/5754/84 PB2 full length product of 986 bp, clones 5, 9 and 10.

DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length which was expected to be 986 bp. Lane 1 consisted of 2 kb DNA Hyperladder II. Lane 2 showed negative control, contained EcoRI enzyme without plasmid DNA. Lane 3 consisted of positive control, contained plasmid DNA

Table 2 Neuraminidase (NA) Primers

ID	Oligo	Sequence 5' – 3'	Product (bp)		
			Start	End	Length
407788	8F	GRA CHC ARG ART C5K MRT G	690	909	219
407793	10F	TGY AGR GAY AAY TGG M55 GG	890	1243	353
407785	5F	CAY DS5 AAT GR5 ACM RT5 MA5 GA	449	909	460
407787	10R	CC5 5KC CAR TTR TCY CTR CA	690	909	219
407792	11R	CCD ASA RTA 5CC 5GA CCA RT	890	1243	353

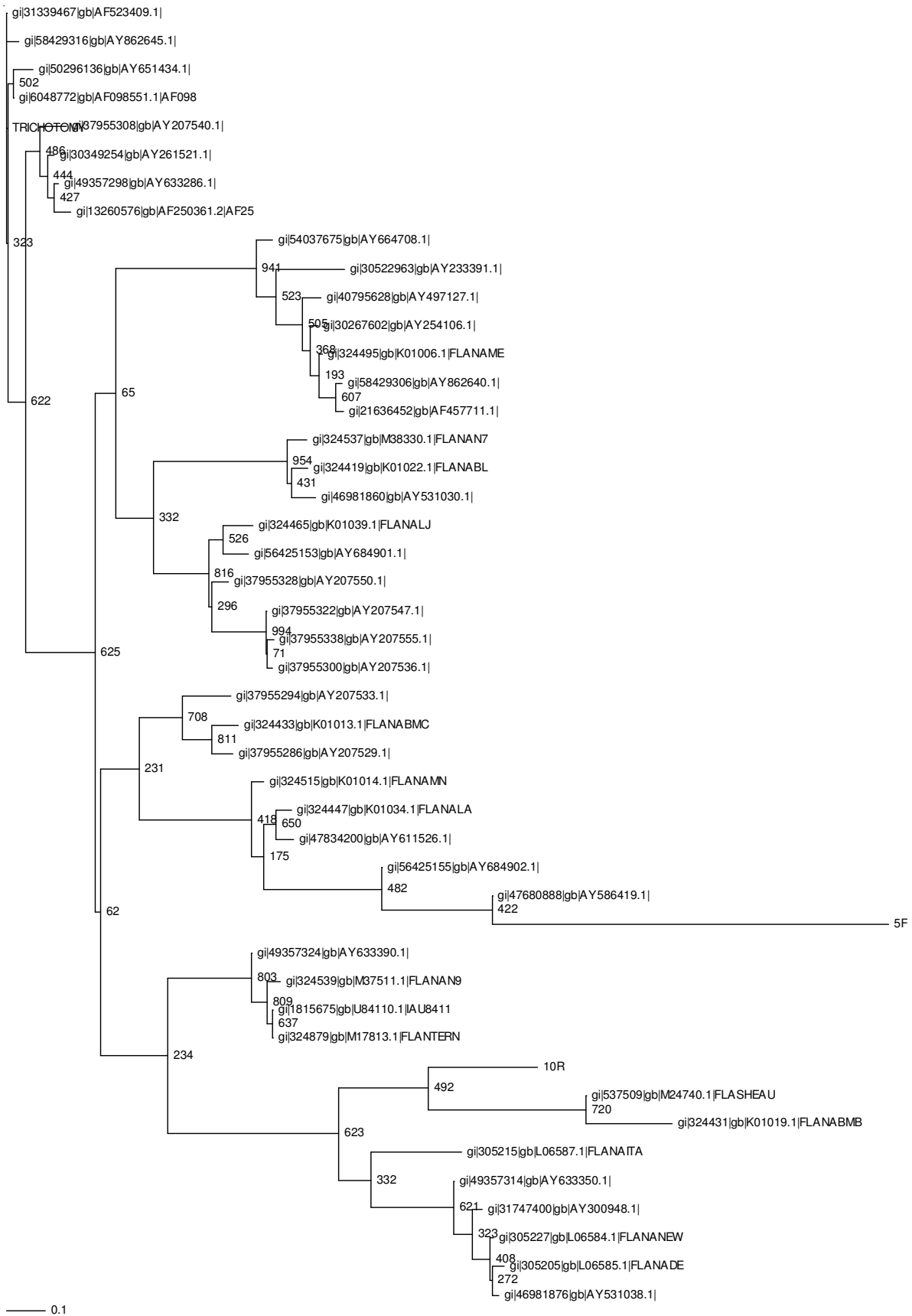


Fig 1 Phylogenetic Tree including 5F, 10R NA Primers and virus subtypes.

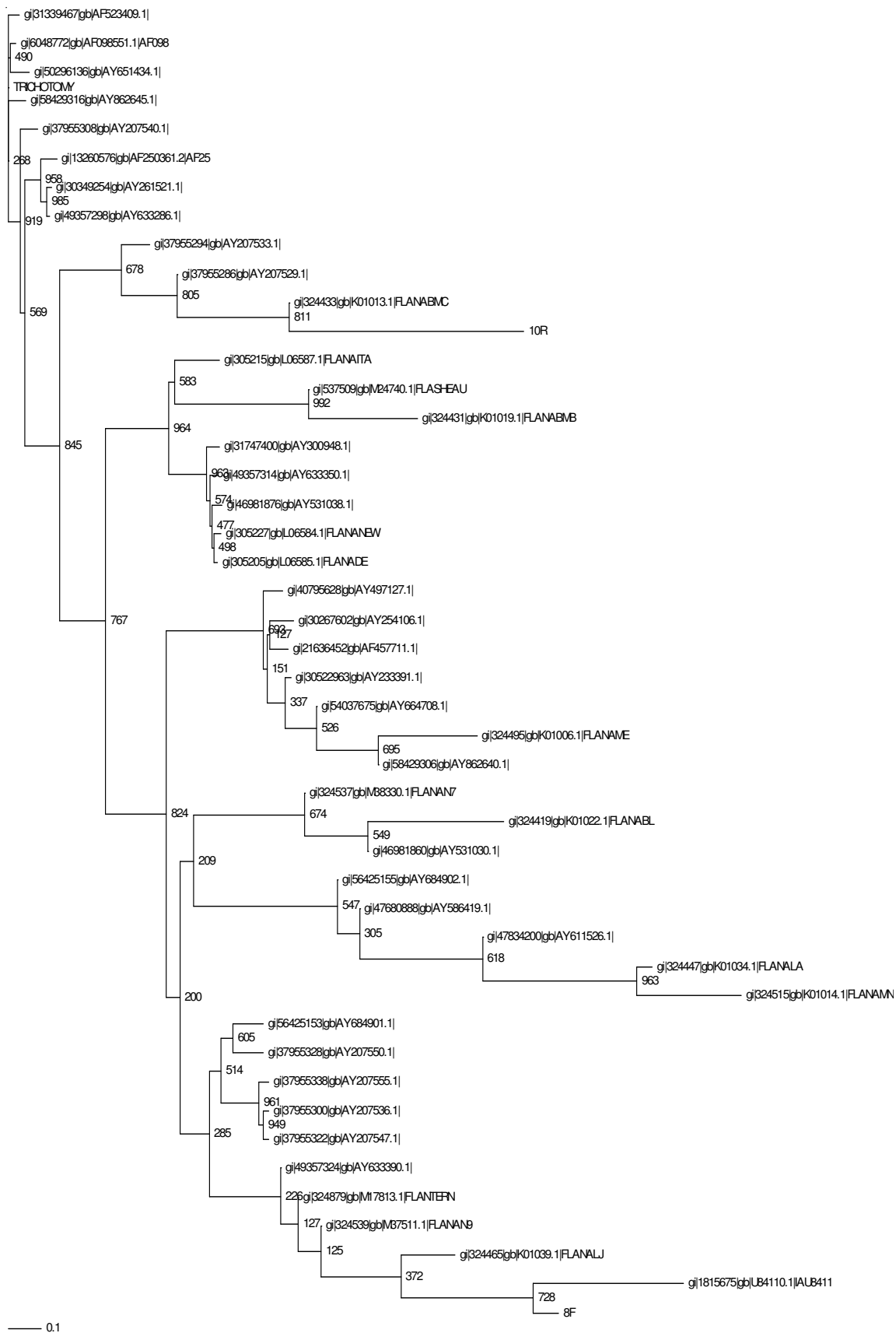


Fig 2 Phylogenetic Tree including 8F, 10R NA primers and virus subtypes.

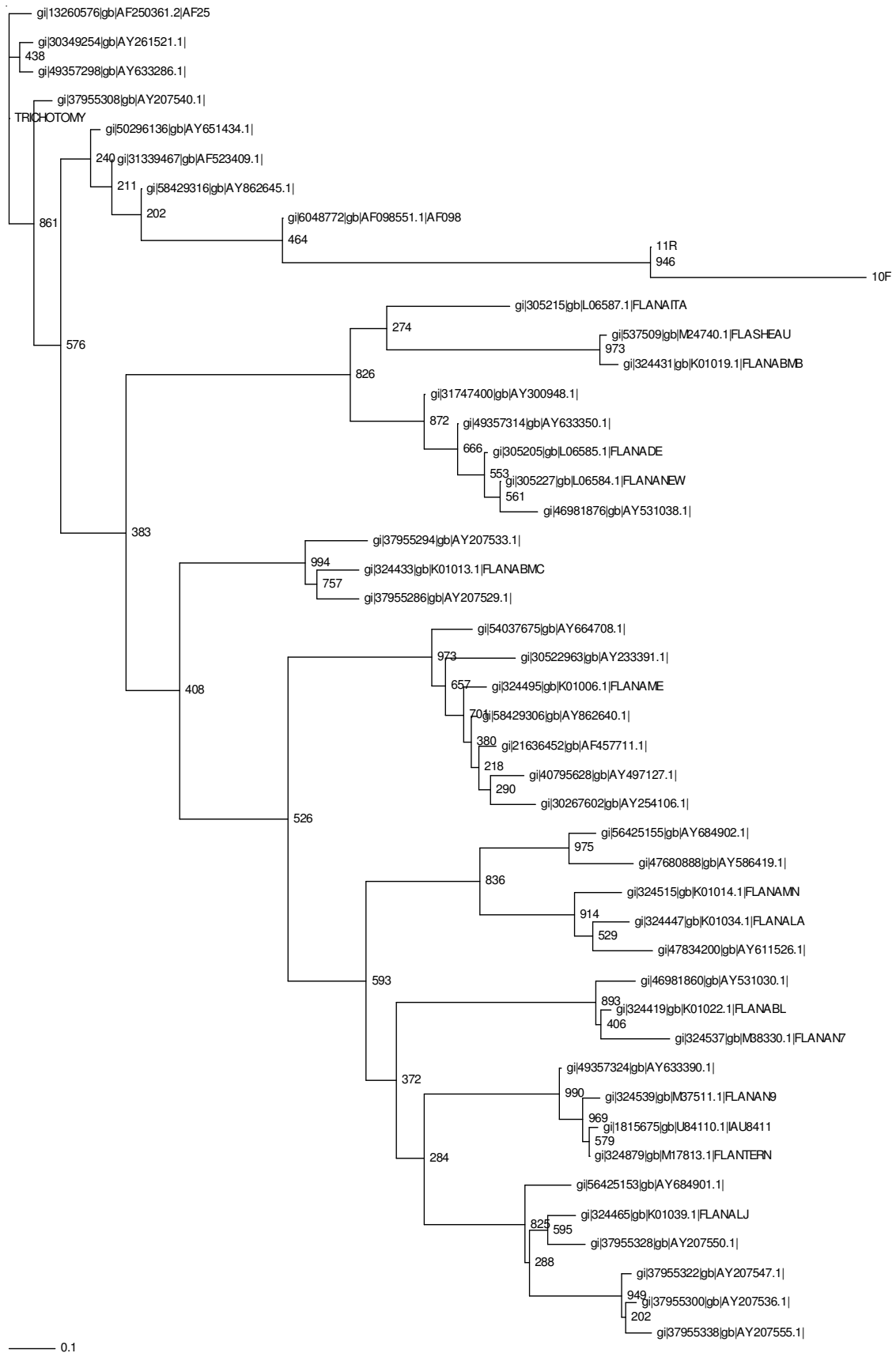


Fig 3 Phylogenetic Tree including 10F, 11R NA Primers and virus subtypes.

without EcoRI enzyme. Lane 4-13 each denoted p-GEM-T easy vector DNA length product of 3015 bp and H7N7/A/Chicken/Victoria/1/85 PB2 full length product of 986 bp, clones 1-10.

DNA products generated by PCR digestion showed plasmid DNA product length of 3015 bp and insert DNA clone product length which was expected to be 986 bp. Lane 1 consisted of 2 kb DNA Hyperladder II. Lane 2 showed negative control, contained EcoRI enzyme without plasmid DNA. Lane 3 consisted of positive control, contained plasmid DNA without EcoRI enzyme. Lane 4-9 each denoted only p-GEM-T easy vector DNA length product of 3015 bp and without any insert DNA clones 1-6.

Determination of PB2 Gene Concentration. Plasmid purification using QIAprep Spin MiniPrep Kit (Qiagen) followed by restriction endonuclease and gel analysis (Fig 4-9) indicated that ligations was highly efficient with most putative PB2 colonies proving to be positive. The presence of PB2 genes in plasmids of isolated clones were confirmed by DNA purity and concentration via Nanodrop Ultraviolet (UV)/Visible Spectrophotometer. DNA purity was estimated with the assumption that pure DNA sample has an absorbance at $A_{260/280}$ of 1.8-2.0. The results showed PB2 gene H1N9 clone 7, PB2 gene H6N5 clone 3 and 6, PB2 gene H15N9 clone 1, PB2 gene H5N3 clone 5 and PB2 gene H7N7 clone 5 were less than 1.8 indicating the DNA samples were not pure because of protein contaminations during plasmid DNA purification procedures while the other clones were pure as expected to be between 1.8 and 2.0 (Table 4).

PCR Amplification of NA Genes. To generate neuraminidase (NA) gene segment samples via PCR amplification (Table 5) was essential for analysis of primer annealing, mutations and phylogeny work. The genes were

need to be isolated, amplified, analyzed then further work including sequencing.

Extraction of DNA Fragments from Agarose Gels. The MinElute Gel Extraction Kit Protocol using a microcentrifuge (Qiagen) was utilized for the isolation of specific DNA

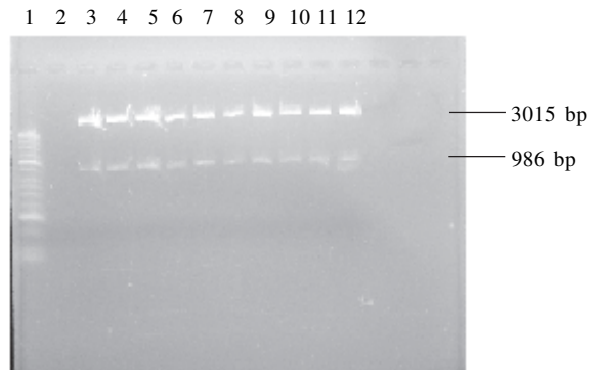


Fig 6 Analysis of H11N6 PB2 gene segment DNA PCR product.

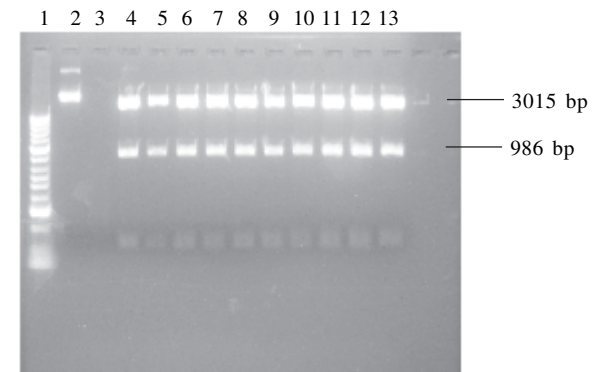


Fig 7 Analysis of H15N9 PB2 gene segment DNA PCR product.

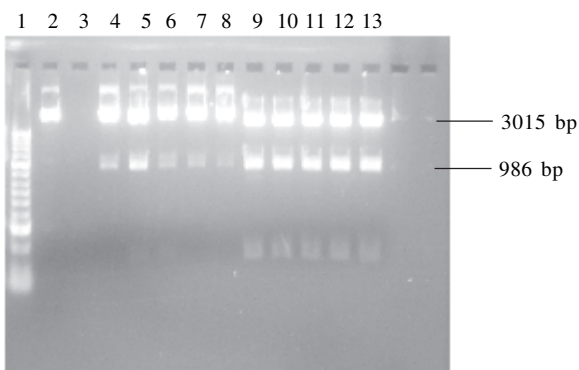


Fig 4 Analysis of H5N3 PB2 gene segment DNA PCR products.

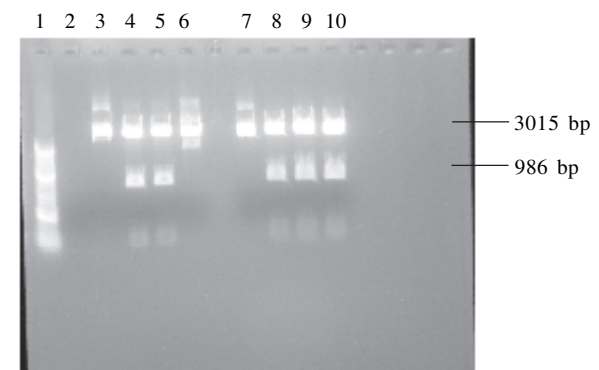


Fig 8 Analysis of H7N7 and H12N9 PB2 gene segment DNA PCR product.

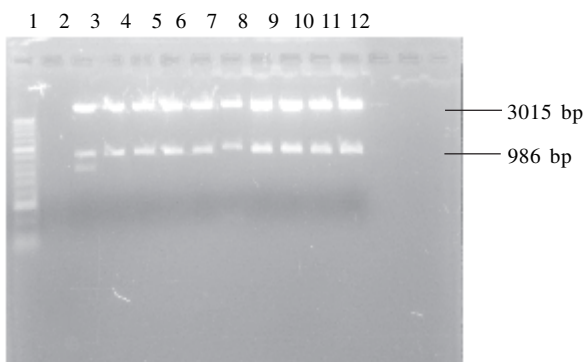


Fig 5 Analysis of H6N5 PB2 gene segment DNA PCR product.

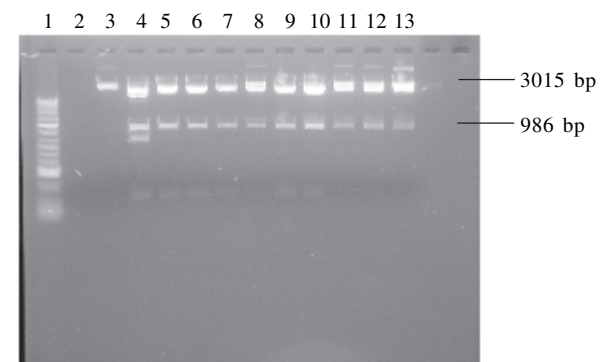


Fig 9 Analysis of H7N7 PB2 gene segment DNA PCR product.

Table 4 DNA purity and concentration of PB2 gene segment clones

CLONE (n = 80)	DNA PURITY (260/280 = 1.8-2.0)	DNA CONCENTRATION (ng μL^{-1})
PB2, H1N9 clones 2, 3, 4, 5, 6, 7, 9, 10 PB2, H3N8 clones 1-9	Clones 2, 3, 4, 5, 6, 9, 10 are good except clone 7 (1.38). H3N8 clones 1-9 are all good.	Clone 2 is the lowest (81.2), clone 7 is the highest (119.2) H3N8 clone 2 is the lowest (109.9), clone 9 is the highest (140.3).
PB2, H6N5 clones 1,2,3,4,6 PB2, H6N5 clones 1-10	H6N5 clones 1-4 are good except clone 6 (1.71). H6N5 clones 1, 2, 4-10 are good except clone 3 (1.57).	H6N5 clone 6 is the lowest (14.0), clone 1 is the highest (247.1). H6N5 clone 1 is the lowest (63.4), clone 10 is the highest (201.2).
PB2, H11N6 clones 1-10	H11N6 clones 1-10 are all good.	H11N6 clone 8 is the lowest (52.4), clone 3 is the highest (92.1).
PB2, H15N9 clones 1-10	H15N9 clones 2-10 are good except clone 1 (1.78).	H15N9 clone 2 is the lowest (74.2), clone 9 is the highest (121.8).
PB2, H5N3 clones 1-10	H5N3 clones 1-4, 6-10 are good except clone 5 (1.75).	H5N3 clone 3 is the lowest (86.8), clone 6 is the highest (153.5).
PB2, H7N7 clones 1-3 PB2, H7N7 clones 1-10	H7N7 clones 1, 3 are good except clone 2 (1.78). H7N7 clones 1-10 are all good.	H7N7 clone 2 is the lowest (95.8), clone 1 is the highest (115.2). H7N7 clone 8 is the lowest (94.4), clone 7 is the highest (155.1).
PB2, H12N9 clones 5,9,10 PB2, H12N9 clones 1-6	H12N9 clones 5,9,10 are all good. H12N9 clones 1-6 are all good.	H12N9 clone 5 is the lowest (98.9), clone 10 is the highest (124.4). H12N9 clone 4 is the lowest (29.3), clone 6 is the highest (150.9).

Table 5 Expected bands for PCR products

Sample to run	Pair of Primers	Product length (bp)	Results from gels (figures were unavailable)
H6N5A/Shearwater/Aust/72	5F + 10R	460	3 Bands: 200 bp, 300 bp, 400 bp
H6N5A/Shearwater/Aust/72	8F + 10R	219	219 bp
H6N5A/Shearwater/Aust/72	10F + 11R	353	353 bp
H6N5A/Shearwater/Aust/72	8F + 9R	169	170 bp
H7N7A/Chicken/Victoria/1/85(POSITIVE CONTROL)	10F + 11R	353	353 bp
H2O(NEGATIVE CONTROL)	No primer	No bands	No bands

fragments from varying weight of agarose gels to have eluted DNA samples for DNA sequencing and testing primer annealing. We had 15 μL elute DNA of each sample resulted from this work and had more 8 μL elute DNA from second clean up of elute DNA extraction (Table 6).

Basic Local Alignment Search Tool (BLAST) Searches. BLASTn was utilized for the alignment of all sequences of NA gene primers generated against a genbank consensus Avian Influenza Virus subtypes. BLAST analysis formed a robust alignment with the consensus sequence indicating 100% homology between 8F, 10F, 11R primers and Influenza A virus subtypes. In particular, 8F primer was labeled in the alignment to correspond with other virus origin depicted 94% homology. It may be noted that 5F primer appeared to be non-specific primer for all virus subtypes available on genbank. Further confirmation of sequence was required

Table 6 MinElute DNA from gel extraction

Sample	Band (bp)	Weight of agarose gel (g)
H6N5 A/Shearwater/Aust/72	360	0.2
H6N5 A/Shearwater/Aust/72	200	0.3
H6N5 A/Shearwater/Aust/72	219	0.3
H6N5 A/Shearwater/Aust/72	300	0.3
H6N5 A/Shearwater/Aust/72	353	0.2
H6N5 A/Shearwater/Aust/72	353	0.4
H6N5 A/Shearwater/Aust/72	169	0.2
H7N7 A/Chicken/Victoria/1/85	353	0.3
H12N9 A/RNStint/WA/5754/84	219	0.2
H12N9 A/RNStint/WA/5754/84	353	0.2
H12N9 A/RNStint/WA/5754/84	460	0.2

using the outline in Table 7.

Phylogenetic Tree Development. Utilizing Clustal-X 1.83 for multiple alignments, it was possible to test variability for regions of 44 different AIV subtype sequences divided into 9 varied NA genes and bounded by NA pair of primer PCR products (Table 8). All trees developed underwent bootstrapping and Treeview programs to distinguish phylogenetic relationships between AIV subtypes and NA primers which randomly align into 0.1 distance matrices of 1000 frequency of each node or label. It was essential to analyze the bootstrap values and positions of sequences that were aligned or matched as well as mismatched or creating a gap (Fig 1-3).

Mutations in NA Primer PCR Products. The information shown in this table was based on scientific findings from Brown *et al.* (1998); Herlocher *et al.* (2003); Herlocher *et al.* (2004); De Jong *et al.* (2005); Jackson *et al.* (2005); Mai Le *et al.* (2005); Jackson *et al.* (2008); Stouffer *et al.* (2008). As can be seen, clinical mutations occurring in 8F and 10R pair of primers at protein positions 274 (820-830 amino acid positions) and 292 (870-880 amino acid positions) and 10F and 11R pair primers at protein positions 294 (880-890 amino acid positions).

DISCUSSION

Amplification of PB2 Gene H1N9/A/Shelduck/WA/1757/78 and H3N8/A/Avian/669/WA/78 via Cloning into *Escherichia coli* Strain DH5 α . Ligation PB2 gene into *E. coli* DH5 α cells were failed because *E. coli* DH5 α cells could

Table 7 BLAST result homology % between NA primers and virus origin

Primer	Nucleotide	Result homology %
5F	CAY DS5 AAT GR5 ACM RT5 MA5 GA (192 redundancy, 48% recombination)	100% for other viruses and bacteria, such as HIV-1, Herpesvirus, Bacillus, Canine distemper, Streptococcus so 5F primer was non-specific primer for Influenza virus.
8F	GRA CHC ARG ART C5K MRT G (192 redundancy, 42% recombination)	100% for H1N2, H3N2, H7N2, H4N6, H7N7, H5N7 94% for H4N6, H1N3, H3N3, H9N2, H6N6, H13N3, H3N2, H3N3, H1N2, H9N2, H7N3, H5N8, H3N6.
10F	TGY AGR GAY AAY TGG M55 GG (32 redundancy, 35% recombination)	100% for H3N2, H1N2.
10R	CC5 5KC CAR TTR TCY CTR CA (32 redundancy, 35% recombination)	100% for H3N1, H11N1, H1N1, H9N1, H13N9, H11N9, H5N1, H2N1, H7N1, H2N9.
11R	CCD ASA RTA 5CC 5GA CCA RT (24 redundancy, 30% recombination)	100% for H7N2, H6N2, H11N2, H9N2, H5N2.

Table 8 Sequence names for clustalX 1.83 (shown in Fig 1-3)

Na subtype	Sequence names of 44 different subtypes
N1	A/Dk/Indonesia/MS/2004(H5N1), A/duck/Korea/S17/03(H6N1), A/turkey/Ontario/HR2/2000(H7N1), A/Aquatic Bird/Hong Kong/m603/98 (H11N1), A/Duck/Shantou/2030/00(H9N1), A/Duck/Ohio/118C/93 (H1N1), A/mallard/Alberta/47/98(H4N1), A/mallard/Stralsund/41-6/81(H2N1).
N2	A/Mallard/New York/6750/78 (H2N2), A/chicken/Guatemala/194573/02 (H5N2), A/duck/Korea/S7/03(H3N2), A/chicken/HongKong/TP38/03(H9N2), A/chicken/California/139/01(H6N2), (A/avian/NY/76247-3/00(H7N2), A/duck/NC/91347/01(H1N2).
N3	A/chicken/British Columbia/04(H7N3), A/Blue-winged Teal/604/78 (H2N3), A/Shearwater/Australia/75 (H5N3), A/black-headed gull/Sweden/2/99(H16N3), A/tern/Astr/775/83(H3N3)
N4	A/gray teal/Australia/2/79(H4N4), A/ruddy turnstone/Delaware/67/98(H12N4), A/Turkey/Ontario/6118/68 (H8N4)
N5	A/Shearwater/72 (N5), A/shearwater/australia/72 (H6N5)
N6	A/mallard/Alberta/42/77(H1N6), A/duck/England/56 (H11N6), A/duck/NewZealand/31/76(H4N6), A/turkey/Minnesota/957/80(H6N6), A/black-headed gull/Sweden/1/99(H13N6), A/pintail duck/Alberta/712/80(H3N6)
N7	A/chick/n/germany/49 (H10N7), A/FPV/Weybridge (H7N7), A/Mallard/64650/03(H5N7)
N8	A/Turkey/Canada/63(H6N8), A/Quail/Italy/1117/65 (H10N8), A/Guinea fowl/New York/4-3587/84 (H3N8), A/Herring gull/DE/677/88 (H2N8), A/pintail/Alberta/207/99(H4N8), A/duck/NY/191255-59/02(H5N8)
N9	A/tern/Australia/G70C/75 (H11N9), A/ruddy turnstone/NJ/60/85 (N9), A/NWS/33HA-A/tern/Australia/G70C/75NA (H1N9), A/teal/Alberta/16/97(H2N9)

Note: H11N6 and H10N8 created gaps in NA pair of primers (see Fig 1-3).

not be competent cells indicating mid-log suspension culture of OD₆₀₀ for 3-4 hr was 1.3 (OD₆₀₀ should be 0.8-1.2 means 8-12.10⁸ cells mL⁻¹). Thereby, low efficiency competent cells (< 1 x 10⁸ cfu µg⁻¹ DNA) showed many cells died because of lack of food source and lots of toxic metabolites. In addition, we applied improperly less incubation time (1-4 h at room temperature) for optimal ligation. As a result, the transformation efficiency (TE) for *E. coli* dH5α cells was either low (T.E. was less than 1 x 10⁹ cfu µg⁻¹ supercoiled plasmid) or the cells did not grow well on agar plates. Consequently, we used another type of competent cells such as *E. coli* Top10 Cells from Invitrogen and the cloning experimentation was performed according to the manufacturer's instructions.

Amplification of PB2 Gene Segment DNA via Cloning into Top10 Cells. Generation of clones was required for future sequencing of the PB2 gene 6 Avian Influenza Virus (AIV) subtypes provided (H5N3, H6N5, H7N7, H11N6, H12N9 and H15N9 subtypes shown in Table 1). DNA cloning procedures including ligations, transformations, plasmid purifications and restriction enzyme digestions were mostly successful with insert DNA bands of the correct size resulting upon agarose electrophoresis analysis. UV analysis for other subtypes identified insert DNA products of expected sizes (full length

PB2 genes were detected at the large 986 bp product) shown in Fig 4-9. However, no visible bands of insert DNA for PB2 gene H12N9 clones 1-6 (Fig 10) appearing upon gel analysis of a potential problems with DNA samples following failed transformation with a lack of white bacterial colony growth on spread agar plates indicating DNA absence. The transformation efficiency (TE) of PB2 gene H12N9 was low (TE = 8 x 10⁶ transformants/µg plasmid DNA while TE of these cells should be ≥ 1 x 10⁹ cfu µg⁻¹ supercoiled plasmid).

Determination of PB2 Gene Concentration. DNA concentration (Table 4) was determined with the assumption

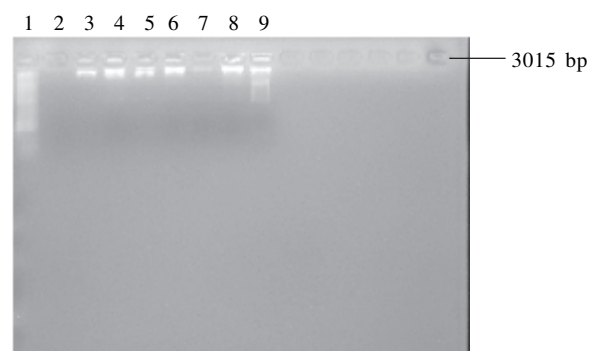


Fig 10 Analysis of H12N9 PB2 gene segment DNA PCR product.

that double stranded DNA sample has a calculation absorbance from 2 to 100 ng μL^{-1} with upper limit to detect 3700 ng μL^{-1} . The results indicated that H6N5 clone 6 and H12N9 clone 4 had much lower concentration than other clones (the concentrations were less than 30 ng μL^{-1}). Consequently, these DNA sample may not be detected by gel electrophoresis after the utilization of restriction enzyme digestions. The determination of DNA concentration will be important for DNA dilutions of future work such as DNA sequencing, DNA annealing with universal HA primer designs, and DNA sampling to detect new diagnostic tools of influenza infection, virulence and pathogenesis.

PCR Amplification of NA Genes. Subtypes NA gene H6N5/A/Shearwater/Aust/72 were annealing with 8F and 10R pair of primers showing expected band of 219 bp, and 10F and 11R pair of primers indicating desired band of 353 bp. On the other hand, NA gene H6N5/A/Shearwater/72 was less accurate annealing with 5F and 10R pair primers with varying bands of 200 bp, 300 bp and 400 bp (Table 5). It is also important to note that 5F and 10R pair of primers was not specific primers for Influenza A viruses as BLAST result homology 100% shown in Table 7. Another subtype NA gene H7N7/A/Chicken/Victoria/1/85 used as positive control was annealing with 10F and 11R pair primers denoting expected band to be 353 bp.

Extraction of DNA Fragments from Agarose Gels. NA gene subtype H6N5/A/Shearwater/72 was successfully extracted from isolation of expected band to be 219 bp indicating matched annealing with 8F and 10R primers and another band of 353 bp showing correct annealing with 10F and 11R primers. Specific DNA fragment of desired band (353 bp) was extracted from NA gene H7N7/A/Chicken/Victoria/1/85 showing annealing with 10F and 11R pair of primers. Another subtype was NA gene H12N9/A/RNStint/WA/5754/84 denoting expected bands of each 219 bp for 8F and 10R primer annealing, 353 bp for 10F and 11R primer annealing and 460 bp for 5F and 10R primer annealing.

Basic Local Alignment Search Tool (BLAST) Searches. It can be shown that 5F primer had 192 redundancies and 48% recombination and when 5F primer paired with 10R primer which had 32 redundancies and 35% recombination, the multiplicity of 5F and 10R pair of primers expected to be 6144 with 83% recombination. It may be noted that 8F primer had 192 redundancies and 42% recombination and when 8F primer paired with 10R primer which had 32 redundancies and 35% recombination, the multiplicity of 8F and 10R pair of primers expected to be 6144 with 77% recombination. Of interest was 10F and 11R pair of primers that 10F primer had 32 redundancies with 35% recombination and 11R primer had 24 redundancies and 30% recombination, there by, when they paired the multiplicity became 768 with 65% recombination. The increased redundancy, multiplicity and recombination conferred problems, meaning that large amounts of primers would have to be utilized in order to anneal template DNA samples. Based on the analysis of homology, redundancy, multiplicity and recombination, it appeared that 10F and 11R pair of primers was better than 8F and 11R pair of primers. Moreover, 8F and 11R pair of primers was much better than 5F and 10R pair of primers. For this reason, 10F and 11R pair

of primers and 8F and 10R pair of primers will be important to utilize as universal primers for NA gene AIV subtype detections.

Phylogenetic Tree Development. The first tree produced aligned 5F and 10R pair of primers with AIV subtypes successfully sequenced (Fig 1). Upon analysis, it appeared that bootstrap values of 9 NA gene AIV subtypes ranged between 332 and 954 (from 33.2% to 95.4%) and there was no gap in sequence positions. As a result, N1, N2, N3, N4, N5, N6, N7, N8 and N9 were aligned or clustal within each subtype. In addition, 5F primer seemed to remain similar to N3 subtype with shortest distance of 422 (42.2%) bootstrap values. 10R primer indicated closely linked to N5 appearing shortest distance of 492 (49.2%) bootstrap value.

Looking at AIV aligned subtypes in fig 2, 8F and 10R pair of primers showed bootstrap values ranged between 226 and 992 (from 22.6% to 99.2%) with two gaps in N8 and N6. When analyzing tree in fig 2 consisting of two gaps, clearer pathways emerged with clear indication that N8 (H10N8) was aligned with N5 showing 583 (58.3%) bootstrap value and was also aligned with its own N8 subtype denoting 964 (96.4%) bootstrap value. Moreover, N6 (H11N6) was aligned with N9 indicating 372 (37.2%) bootstrap value and was also aligned with its own N6 subtype appearing 285 (28.5%) bootstrap value. Consequently, only N1, N2, N3, N4, N5, N7 and N9 were aligned within each subtype. Furthermore, 8F primer appeared to remain closely related to N9 with 728 (72.8%) bootstrap value while 10R primer indicated similar to appearing N4 with 811 (81.1%) bootstrap value.

Fig 3 indicated the phylogenetic relationship of NA gene AIV subtypes with 10F and 11R pair of primers. This tree depicted bootstrap values ranged between 825 and 994 (from 82.5% to 99.4%) with a gap in N8. When analyzing this tree consisting of a gap, accurate indication that N8 (H10N8) was aligned with N5 showing 274 (27.4%) bootstrap value and was also aligned with N8 occurring 826 (82.6%) bootstrap value. Consequently, N1, N2, N3, N4, N5, N6, N7 and N9 were aligned within each subtype. Furthermore, 10F and 11R pair of primer linked similarly to N1 with 464 (46.4%) bootstrap value.

The resultant primers were designed in conjunction with high bootstrap value of sequences found in multiple numbers of the NA gene family. That is, 10F and 11R pair of primers showed the highest bootstrap value (99.4%) followed by 8F and 10R pair of primers (99.2%), then, 5F and 10R pair of primers (95.4%). Since sequence alignment was rarely perfect, the primers were degenerate to permit at sequence positions of low matches or creating gaps. Once aligned, regions of high aligned sequences bounded by NA primers were analyzed for the appropriateness for essential primer utilizations. For this reason, 8F and 10R pair of primers and 10F and 11R pair primers will be able to identify variable NA gene families of AIV subtypes concomitantly with the detection of any mutations occurring in these primers.

Mutations in NA Primer PCR Products. Of interest were clinical mutations occurring in 8F and 10R pair of primers at protein positions 274 (820-830 amino acid positions) and 292 (870-880 amino acid positions) and 10F and 11R pair primers at protein positions 294 (880-890 amino acid positions).

Referring to Table 3, these mutations gave rise to antiviral drug resistance (Oseltamivir). It appeared that 8F and 10R primers contained a tyrosine residue at position 274 (Y) indicating that a possible shift from H (histidine) to Y (tyrosine) is required to block neuraminidase inhibitor (Oseltamivir), as well as an arginine (R)-to-lysine (K) change at position 292. It can be noted that 10F and 11R pair of primers had a serine (S) to asparagine (N) substitution at position 294 to confer Oseltamivir resistance. Thus, 8F and 10R pair of primers and 10F and 11R pair of primers consisted of mutations that may have a higher ability to promote virulence as a result of drug resistance pathway.

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