Design of universal primers for the detection of all influenza A viruses in a single enzymatic reaction

by

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Abstract

Influenza A viruses have single-stranded, segmented RNA genome of negative sense. They are subdivided into subtypes based on two proteins located on the surface of the virus: hemagglutinin (HA) and neuraminidase (NA). To date, influenza A viruses representing 18 HA and 11 NA subtypes have been detected in birds and some mammals throughout the world. They undergo high mutation rates in most regions of viral segments, in particular HA and NA genes, leading to difficulty of molecular diagnosis of the virus since the sequences of the primer sets display considerable numbers of mismatches to the sequences of animal influenza A viruses. The aim of this study was to innovate a reliable, simple method for the detection of all subtypes of influenza A viruses in a single enzymatic reaction using a new set of universal primers. Highly conserved regions of the Matrix (M) gene among a panel of different strains of influenza A virus which are available from influenza research database, were used to design the universal primers. The conserved fragment in M gene was determined through gene alignment using Geneious software. In addition, the quality of the designed primers was checked by using another software to determine the proportion of primer-dimer and cross-dimer. A total of 30 swabs were collected from 10 wild ducks, 10 chicken, and 10 humans expected to be infected with influenza. All samples were processed for RNA extraction and gene amplification using RT-PCR technique. Results of primers quality determination showed that both primers (forward and reverse) were excellent and would not cause PCR problems (depending on Delta G values, which were less than -5). In addition, RT-PCR results showed that viral M gene was successfully amplified from most samples that collected from birds and humans. These results confirm that this novel method can be applied to detect any influenza virus subtype originated from any host species.

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List of abbreviations

˚C	Degree Celsius
bp	Base pair
cDNA	Complementary ribonucleic acid
CF	Complement fixation
HA	Hemagglutinin
HI	Haemagglutination inhibition
hr	Hour
M	Matrix
min	Minute
NA	Neuraminidase
NP	Nucleoprotein
NS	Non-structural
PA	Polymerase acidic
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
TBE	Tris/Borate/EDTA
V	Volt

Chapter 1

Introduction and aim

1.1 Influenza A virus

Influenza viruses belong to the *"Orthomyxoviridae"* family and are classified into six different genera: influenza A, influenza B, influenza C, influenza D, Thogotovirus, and Isavirus (Bouvier and Palese, 2008). The most serious types that cause dangerous outbreaks with high morbidity and mortality are influenza A viruses because they mutate more rapidly and have a wider range of hosts (Khanna et al., 2008). Influenza A viruses infect animals, including birds, pigs, horses, and human (Reperant et al., 2009). They are enveloped with surface glycoprotein spikes and a segmented RNA genome of negative sense. RNA of influenza A virus is organized into 8 segments, in total around 13600 nucleotides long (Hoffmann et al., 2001). These are the polymerase basic (PB1 and PB2), the polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non–structural (NS) genes (Samji, 2009) **(Figure 1.1)**.

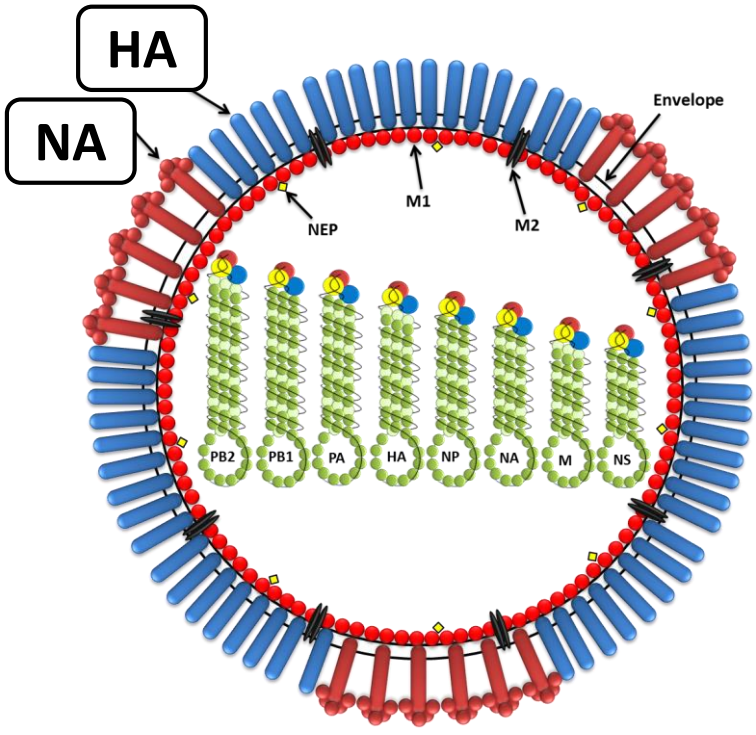

Figure 1.1 Schematic diagram of influenza virus A particle.

The virus has 8 RNA segments and each segment encodes one or more proteins. The envelope contains two types of proteins, the haemagglutinin (HA), neuraminidase (NA).

Both NA and HA genes encode surface glycoproteins and influenza A virus can be classified into several subtypes according to the antigenic diversity of those surface antigens . There are 18 HA and 11 NA subtypes described as H1–H18 and N1–N11 with amino acid sequences differing by 30% or more between subtypes (Tong et al., 2012). Therefore, in theory, 198 different virus subtypes are possible by combinations of these proteins (Yang et al., 2015).

1.2 Genetic variation among influenza viruses

Genetic change in influenza A virus mainly occurs by 'antigenic drift'. This is due to the accumulation of point mutations over time, which results from a lack of proofreading mechanism in the RNA polymerase, leading to incorrect ribonucleotide insertions during replication (Sanjuan and Domingo-Calap, 2016). Such changes occur progressively over a period of time accompanied by a gradual change in surface glycoproteins (HA and/ or NA). All viral segments genetically change over time. Segment 7 (M gene) has been shown more genetically stable in comparison with the other segments (Shao et al., 2017) **(Figure 1.2)**. Bioinformatics of M gene from data available in GenBank shows similarities in some regions of M gene between different virus subtypes originated from different host species.

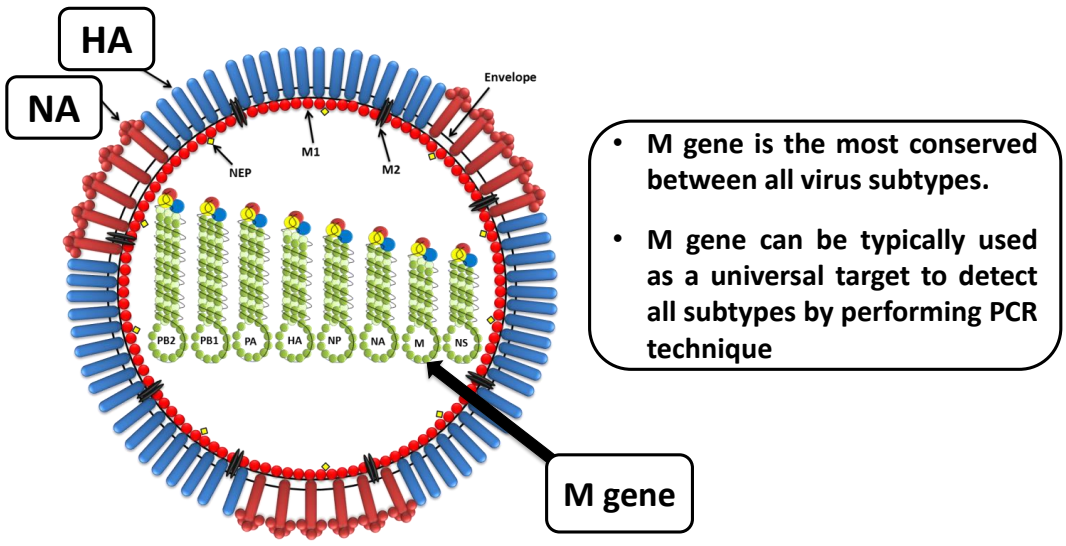

Figure 1.2 The conserved (M) gene of all influenza A viruses.

1.3 Laboratory diagnosis

Laboratory investigations should confirm the suspected cases and differentiate them from flu–like diseases which may be caused by other respiratory viruses including adenovirus, picornaviruses, parainfluenza viruses, respiratory syncytial viruses, rhinovirus, and also by bacterial agents such as chlamydia, legionella and mycoplasma (Koski and Klepser, 2017). Further investigations of influenza virus have been done by nucleic acid testing (RT–PCR), and serological diagnostic tests (complement fixation (CF), haemagglutination inhibition (HI), and neutralization tests). All of these diagnostic tests have different sensitivity rates with some advantages and disadvantages (Dwyer et al., 2006). RT–PCR is generally more sensitive and specific and is not time consuming. It provides accurate detection, and facilitates the typing and subtyping of influenza viruses (Ellis et al., 1997). However, the frequent genetic changes of influenza viruses may result in a false-negative diagnosis of influenza A virus infection by performing the current protocols for PCR analysis (Trevino et al., 2011). Therefore, using a developed PCR protocol for influenza virus detection would be valuable for virus surveillance and screening.

1.4 Aim of the study

The overall aim was to design a pair of universal primers to detect all influenza A virus subtypes that infect different host species through the amplification of a conserved gene fragment. The aim was achieved by the following objectives:

1. To determine the conserved regions in the virus genome through alignment of M genes of different virus species obtained from the available influenza database.

2. To determine the location of the forward and reverse primer in the conserved regions using the Geneious software.
3. To check the designed primers for the secondary structure formation including primer dimer and cross dimer using Oligoanalyzer 3.1 software.
4. To assess the quality and specificity of the designed primers by performing RT-PCR technique on samples collected from chicken, duck, and humans expected to be infected with influenza.

Chapter 2

Materials and methods

2.1 Materials

Materials used throughout the period of this project are as follows:

1. Influenza database website
2. Oligoanalyzer 3.1 software
3. Geneious Inspirational Software for Biologists sofware
4. Swabs
5. Centrifuge tubes
6. Eppendorf tubes
7. Viral RNA extraction kit
8. RT-PCR kit
9. Universal primers
10. Thermal cycler
11. Agarose gel
12. Ethidium bromide
13. TBE buffer
14. Micropipettes and different sizes of tips
15. Gel documentation system
16. Nuclease-free water
17. Phosphate buffer saline
18. Glycerol
19. Microcentrifuge
20. UV hood

2.2 Methods

2.2.1 Design of universal primers

To obtain the complete viral M gene nucleotide sequence, the influenza database website (https://www.fludb.org/brc/home.spg?decorator=influenza) was used for this purpose.

Highly conserved sequences among several strains of influenza A viruses of different host species were used to design the universal primers. A number of influenza virus M gene of different host species (human, chicken, and duck) were aligned together to determine the highly conserved regions using Geneious Inspirational Software for Biologists (www.geneious.com). The site of forward and reverse primers was then initially determined by observing the similarities between nucleotides of the selected virus strains **(Figure 2.1)**.

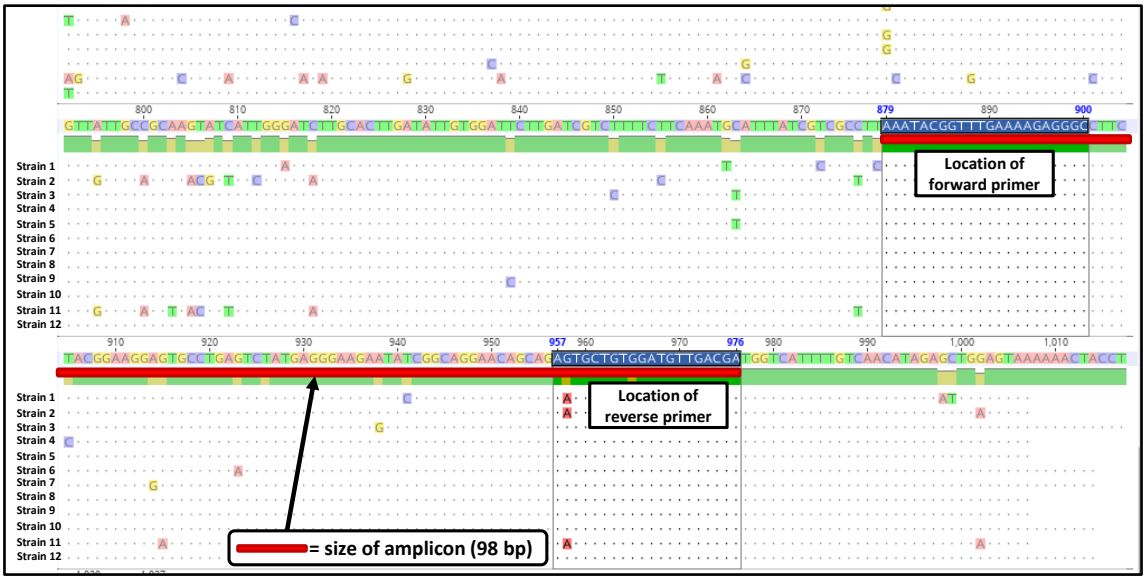

Figure 2.1 Determination of highly conserved nucleotide sequences of influenza virus M gene. Nucleotide sequences of partial viral M gene of different host species (chicken, duck, and human) were aligned to determine the conserved areas that were used for designing the forward and reverse primers. The locations of forward and reverse primers are highlighted in blue, and the expected amplicon size is determined by a red line.

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To check the quality of the forward and reverse primers, an available software program (https://eu.idtdna.com/calc/analyzer) was used for checking the possibilities of a primer dimer, cross dimer. Primers were designed to amplify a partial fragment (98 base pair) of viral M gene **(Table 2.1)**. The primers were supplied by Bioneer Corporation - Daejeon, Korea, Republic of (South Korea).

Table 2.1 Forward and reverse universal primers for influenza virus M gene. This set of primer was designed for the amplification of highly conserved regions of the seventh viral segment (M gene) of influenza A viruses derived from different host species. The expected PCR product (the size of band on agarose gel) is 98 base pair.

Gene	Forward primer	Reverse primer	Amplicon size
M	M-F-879-900 (22 bp) AAATACGGTTTGAAAAGAGGGC	M-R-976-957 (20 bp) TCGTCAACATCCACAGCAYT	98 bp

2.2.2 Sample collection

A total of 30 cloacal or nasal swabs were collected from 10 wild ducks, 10 chicken, and 10 humans expected to be infected with influenza from Basra governorate. Cloacal samples were taken from wild duck, while nasal swabs were taken from chicken and humans **(Figure 2.2)**. Each sample was collected in a sterile phosphate buffer saline (PBS) with glycerol. Samples were kept on ice during collection and then shipped to the laboratory immediately. They were centrifuged at 1000 xg for 10 minutes, and the supernatants were gently collected and moved to new-labelled tubes. They were then directly prepared for viral RNA extraction **(Figure 2.3)**.

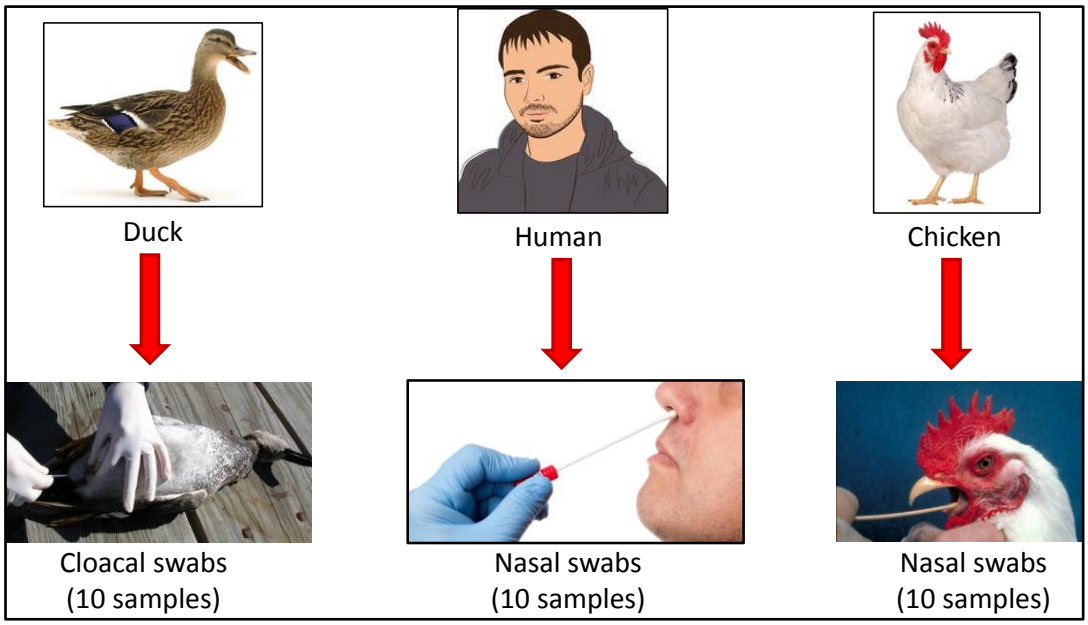


Figure 2.2 Methods of sample collection from human, chicken, and duck. Nasal swabs were collected from human and chicken while cloacal swabs were collected from ducks.

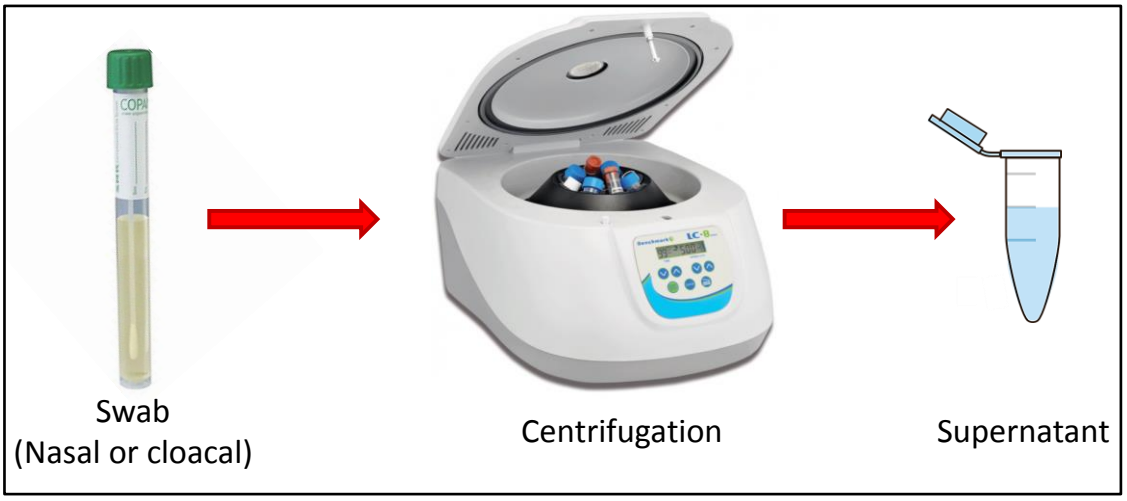


Figure 2.3 Clarification of nasal and cloacal swabs. All swabs were clarified by centrifugation and the supernatants were then collected.

2.2.3 Viral RNA extraction and quantification

Viral RNA was extracted by using a QIAamp viral RNA purification kit (Qiagen) following the manufacturer's instructions. The concentration of purified RNA was determined using NanoDrop spectrophotometer by UV absorption. Eluted viral RNA samples were stored at –20˚C until further use **(Figure 2.4)**.

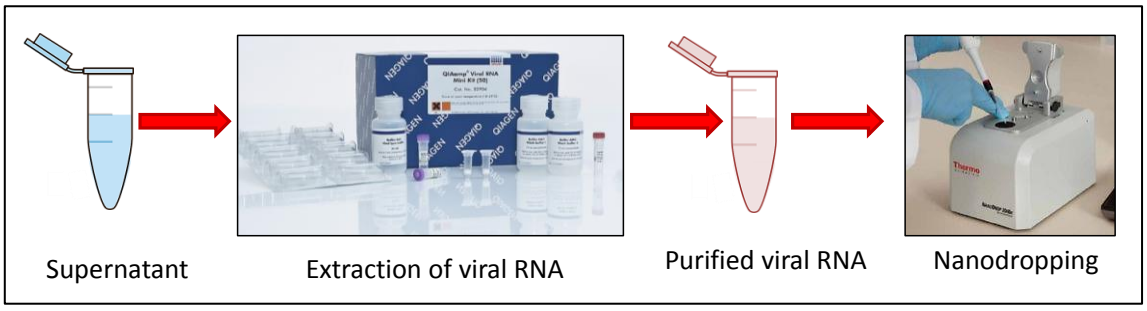


Figure 2.4 Viral RNA extraction and quantification. Viral RNA was extracted from supernatants and quantified by NanoDrop spectrophotometer.

2.2.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

Amplification of a partial region of influenza virus M gene was performed using One Step RT–PCR kit (Bioneer). The designed forward primer 5'-AAATACGGTTTGAAAAGAGGGC -3' and reverse primer 5'-TCGTCAACATCCACAGCAYT -3' were used to amplify 98 base pare (bp)

fragment. Both cDNA synthesis and PCR amplification were performed in a single tube using this system. Starting material of viral RNA used in cDNA synthesis was 100 ng/µl. The RT-PCR conditions were: cDNA synthesis at 45°C for 30 min, initial denaturation at 95°C for 2 min followed by 35 cycles of: denaturation at 95°C for 10 s, annealing at 58°C for 20 s, and extension at 72°C for 30 s. The reaction was then held at 72°C for 5 min, and then cooled down at 4°C for 5 min **(Table 2.2)**.

Table 2.2 RT-PCR conditions for amplifying a partial fragment of influenza virus M gene.

Step	Temperature	Time
cDNA synthesis	45°C	30 minute
Initial denaturation	95°C	2 minute
35 cycles of:		
Denaturation	95°C	10 seconds
Annealing	58°C	20 seconds
Extension	72°C	30 seconds
Final extension	72°C	5 minutes
Cooling down	4°C	5 minutes

2.2.5 Agarose gel electrophoresis

The amplified PCR products were detected using 1.5% agarose gel prepared with agarose in TBE buffer. The mixture was stirred well and melted in a microwave oven, and mixed once or twice during microwaving. The gel was then cooled to 55°C, and 2 µl of ethidium bromide per 50 ml agarose gel was added. The gel was then poured into a gel casting tray, and a 10 well gel comb was inserted, and then left for 30 to 45 min to set. The comb was then carefully pulled out and the gel was placed in the electrophoresis tank. Running buffer was added into the tank up to 2 to 3 mm over the gel. The sample was then loaded on the gel by adding 10 µl of PCR product to each well. A 100 bp DNA ladder was loaded in one of the side well. The lid was placed on the gel box and the electrical current was

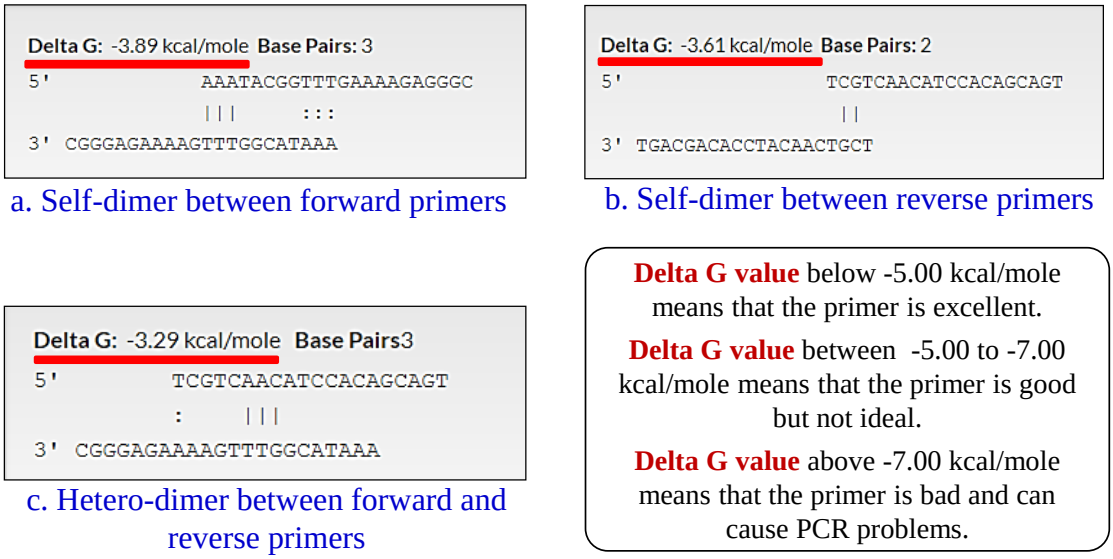
connected for around 1 hr with 90 V. The gel was then carefully removed from the tray and examined under a UV trans–illuminator. The size of the gene was estimated by comparison with the standard DNA ladder.

Chapter 3

Results

3.1 Universal primers efficiency

Although the universal primers were designed from the highly conserved region of viral M gene, secondary structures including primer dimer, cross dimer might form and eventually reduce the efficiency of these primers. Therefore, a software program (https://eu.idtdna.com/calc/analyzer) was used to check the possibilities of forming such secondary structures. The results showed that the ratio of secondary structure in the designed primers (according to the values of Delta G) was very low and would not affect or inhibit gene amplification **(Figure 3.1)**.



a. Self-dimer between forward primers

b. Self-dimer between reverse primers

c. Hetero-dimer between forward and reverse primers

Figure 3.1 Determination of universal primer efficiency. Delta G values of self-dimer between forward primers (a), reverse primers (b), and hetero-dimer between forward and reverse primers (c) were less than -5 which means that all primers are excellent and would not cause PCR problems.

3.2 Detection of influenza A viruses by RT-PCR

Using the designed universal primers, PCR products of partial matrix (M) gene of influenza virus A were separated on 1.5% agarose gel pre-stained with ethidium bromide. Twenty-six samples (8 ducks, 9 chickens, and 9 humans) out of 30 gave positive results on agarose gel. The separation of selected PCR products on agarose gel is shown in **Figure 3.2**.

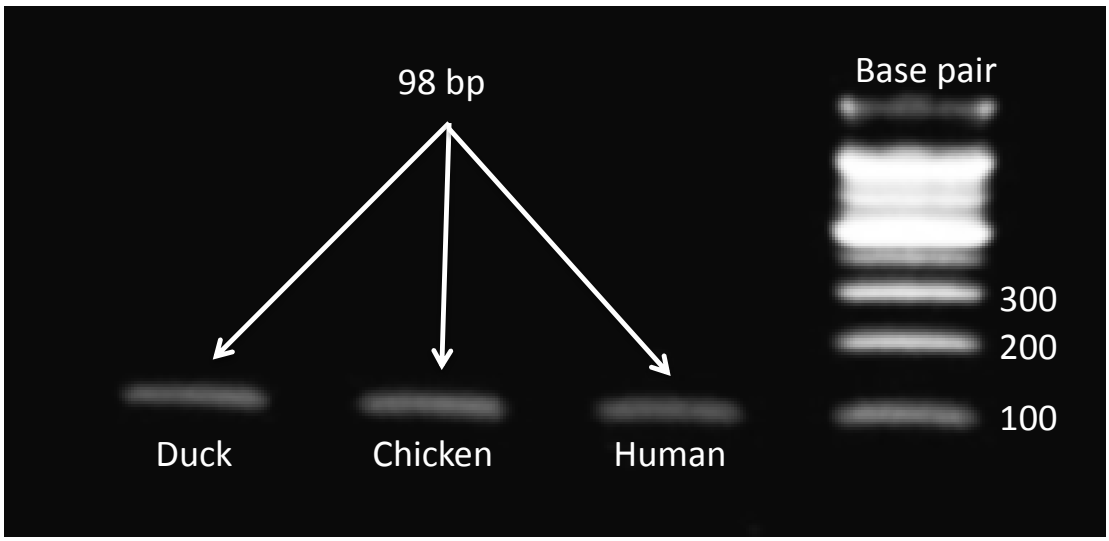


Figure 3.2 Detection of influenza A virus by polymerase chain reaction. PCR product of partial matrix (M) gene of influenza A virus was detected on 1.5% agarose gel pre-stained with ethidium bromide. The results showed the amplification of 98 bp of viral M gene from cloacal swabs collected from ducks and nasal swabs collected from humans and chickens.

Chapter 4

Discussion

Discussion

Influenza virus A has been classified into subtypes according to the variations in the surface hemagglutinin (HA) and neuraminidase (NA) proteins (Gamblin and Skehel, 2010). So far, 18 HA and 11 NA have been circulating in different host species, in particular wild ducks. Each influenza virus particle must have HA and NA, so therefore, 198 different virus subtypes are possibly formed by combinations of these proteins (Mehle, 2014). The sensitivity and specificity of PCR-based methods for the detection of virus subtype are most critically determined by the choice of primer sequences. Nucleic acid amplification techniques based on RT-PCR assays are regarded as a specific diagnosis to confirm the influenza virus infection (Fouchier et al., 2000). Although the nucleic acid-based techniques are highly sensitive and specific, the high mutation rate of the influenza RNA-dependent RNA polymerase could limit the utility of the techniques (Kim and Poudel, 2013). The sequences of the primer sets that are routinely used for PCR-based detection of influenza A virus may be appropriate for the detection of some virus strains circulating in domestic poultry and human population. However, the use of the same primer sets over time would play a role in inhibiting virus detection and induction of false-negative results. In addition, false-negative results are also generated when the host is infected with an unknown or unexpected virus subtype.

In this study, an extensive amount of the sequence information available for influenza A virus was used to design a universal PCR primer set for diagnostic purposes. The designed forward and reverse primers span conserved sequences in gene segment 7 of influenza A virus and have no homology to nucleotide sequences from other species available from influenza database. The experimental data gained in this study confirmed that PCR amplification with this set of primers does not have any problems.

This would be owing to the high efficiency of the primers used, which had a low possibility of secondary structure (self-dimer and hetero-dimer).

In conclusion, by performing this novel PCR-based assay, diagnosis of influenza infection with any virus subtype would be achieved within a single PCR tube in a single working day, which would be a significantly faster tool than previous PCR protocols for the diagnosis of bird and human influenza A virus infection.

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الخلاصة

تحتوي فيروسات الأنفلونزا نوع أ على الحمض النووي الرايبوزي (RNA) والذي يكون بشكل مجزأ الى عدد من القطع. تنقسم هذه الفيروسات إلى أنواع فرعية اعتمادا على الاختلافات في تركيب اثنين من البروتينات الموجودة على سطح الفيروس: هيماغلوتينين (HA) ونيورامينيديز (NA). حتى الآن ، يوجد 18 نوعا من الـHA و 11 نوعا من الـNA تتواجد في الطيور وبعض الثدييات المنتشرة في جميع أنحاء العالم. تخضع فيروسات الانفلونزا لمعدلات طفرات عالية في معظم مناطق الحمض النووي الرايبوزي ، وعلى وجه الخصوص جينات HA و NA ، مما يؤدي إلى صعوبة التشخيص الجزيئي للفيروس باستخدام تقنية RT-PCR لأن تسلسلات القواعد النتروجينية لمواقع الارتباط بين البرايمرات والحمض النووي ممكن ان تتغير بمرور الوقت بسبب هذه الطفرات. الهدف من هذه الدراسة هو ابتكار طريقة مبسطة وموثوقة للكشف عن جميع الأنواع الفرعية لفيروسات الإنفلونزا A في تفاعل إنزيمي واحد باستخدام مجموعة جديدة من البرايمرات. لتصميم هذه البريمرات، تم تحديد مناطق ذات تشابه كبير من جين الماتريكس (M) بين مجموعة من سلالات مختلفة من فيروس الأنفلونزا أ والتي تتوفر من قاعدة بيانات أبحاث الأنفلونزا. تم تحديد الجزء المتشابه في الجين M من خلال محاذاة الجينات باستخدام برنامج Geneious. بالإضافة إلى ذلك، تم فحص جودة البريمرات المصممة باستخدام برنامج آخر لتحديد احتمالية حدوث ارتباطات غير مرغوب بها بين البريمرات. تم جمع 30 مسحة من 10 بطات برية و 10 دجاجات و 10 أشخاص من المتوقع أنهم مصابون بالأنفلونزا. تم تمرير جميع العينات لاستخلاص الحمض النووي الريبوزي وتضخيم الجينات باستخدام تقنية RT-PCR. أظهرت نتائج تحليل الجودة للبريمرات أن كلا البرايمرين (الامامي والعكسي) كانت ممتازة ولن تتسبب في حدوث مشاكل خلال عملية الـ PCR (اعتمادا على قيم دلتا G ، التي كانت أقل من -5). بالإضافة إلى ذلك، أظهرت نتائج RT-PCR أن الجين M قد تم تضخيمه بنجاح من معظم العينات التي تم جمعها من الطيور والانسان. تؤكد هذه النتائج أنه بتطبيق هذه الطريقة المبتكرة، وبالرغم من حدوث الطفرات المتكررة لفيروس الانفلونزا، سيتم الكشف عن أي نوع فرعي لهذه الفيروسات في اختبار انزيمي واحد دون الحاجة الى اتباع الطرق التقليدية للتحري عن وجود الفيروس.

تصميم بريمرات شاملة للكشف عن جميع فيروسات الانفلونزا نوع أ في تفاعل انزيمي واحد

بحث مقدم من قبل الطالب

اسعد رشيد حامد

المرحلة الخامسة

اشراف

الدكتور فراس طه منصور

نيسان/ 2018