Design of universal primers for the detection of Newcastle disease viruses in a single enzymatic reaction

by

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Abstract

Newcastle disease viruses have single-stranded, non-segmented RNA genome. They are subdivided into different strains based on the pathogenicity of the virus. The viral genome is not identical between these strains, and thus the molecular diagnosis requires using a specific pair of primers to amplify a specific region in the genome of each virus strain. The aim of this study was to innovate a reliable, simple method for the diagnosis of any suspected infection with Newcastle disease virus in a single enzymatic reaction using a new pair of universal primers. Highly conserved regions of the viral matrix (M) gene among a panel of different strains of Newcastle disease virus which are available in NCBI database, were used to design the universal primers. The conserved fragment in M gene was determined through gene alignment using Geneious Inspirational Software for Biologists. 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 20 oropharyngeal swabs were collected from 10 pigeons and 10 chickens showing signs of Newcastle disease. All samples were processed for RNA extraction and gene amplification using conventional and Real-Time RT-PCR techniques, followed by gene sequencing and bioinformatics. The quality of 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, the results of the conventional and Real-Time RT-PCR showed that viral M gene was successfully amplified from almost all samples that were collected from pigeons and chickens, and this was also confirmed by gene sequencing and bioinformatics. These findings confirm that this novel pair of universal primer can be used to detect and quantify any Newcastle disease virus originated from any host species in a single enzymatic reaction.

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List of abbreviations

bp	Base pair
˚C	Degree Celsius
cDNA	Complementary ribonucleic acid
F	Fusion
HN	Hemagglutinin- neuraminidase
hr	Hour
L	Polymerase
M	Matrix
min	Minute
NCBI	National Center for Biotechnology Information
ND	Newcastle disease
NDV	Newcastle disease virus
NP	Nucleocapsid
P	Phosphoprotein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
TBE	Tris/Borate/EDTA
UV	Ultraviolet
V	Volt

Chapter One

Introduction and aim

1.1 Newcastle disease

Newcastle disease (ND) is one of the most deadly diseases of poultry around the globe (Shabbir *et al.*, 2013). It is a worldwide problem that presents primarily as an acute respiratory disease, but depression, nervous manifestations, or diarrhea may be the predominant clinical form. Severity of the disease depends on the virulence of the infecting virus and host susceptibility (Alexander, 2000).

ND is transmitted most often by direct contact with diseased or carrier birds. Infected birds may shed the virus in their feces, contaminating the environment. Transmission can then occur by direct contact with feces and respiratory discharges or by contaminated food, water, equipment, and human clothing. Newcastle disease viruses can survive for several weeks in the environment, especially in cool weather (Shankar, 2008).

1.2 Newcastle disease virus

Newcastle disease is caused by Avian Paramyxoviruses type-1 (APMV-1) which is classified with the other avian paramyxoviruses in the genus *Avularius*, family *Paramyxoviridae*. The members of this family consists of no segmented, enveloped RNA viruses with helical capsid symmetry (Yoshida and Samal, 2017).

The envelope of the virion is derived from the host cell plasma membrane with an outer surface consisting of two viral glycoproteins: fusion (F) protein, and hemagglutinin-neuraminidase (HN) protein. The fusion (F) protein functions for the fusion of viral envelope with the host cell membrane and the HN protein is responsible for the attachment of the virion to the host cell receptor (Takimoto *et al.*, 2002). The helical nucleocapsid of core of the virion acts as a template for RNA synthesis all

the time. The core consists of nucleocapsid (NP) proteins tightly bound to the genomic RNA. Polymerase (L) protein is also attached to them. In between the viral envelope and nucleocapsid core is another layer of protein, the matrix or M protein. This protein acts as a driving force for the assembly of the virus particles (Sonali, 2018).

The genome of Newcastle disease virus consists of six genes which code for six different proteins. The genes arranged in tandem in order of 3'-NP-P-M-F-HN-L-5' encode for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L), respectively (Yu *et al.*, 2017).

Figure 1-1 shows the typical structure of Newcastle disease virus.

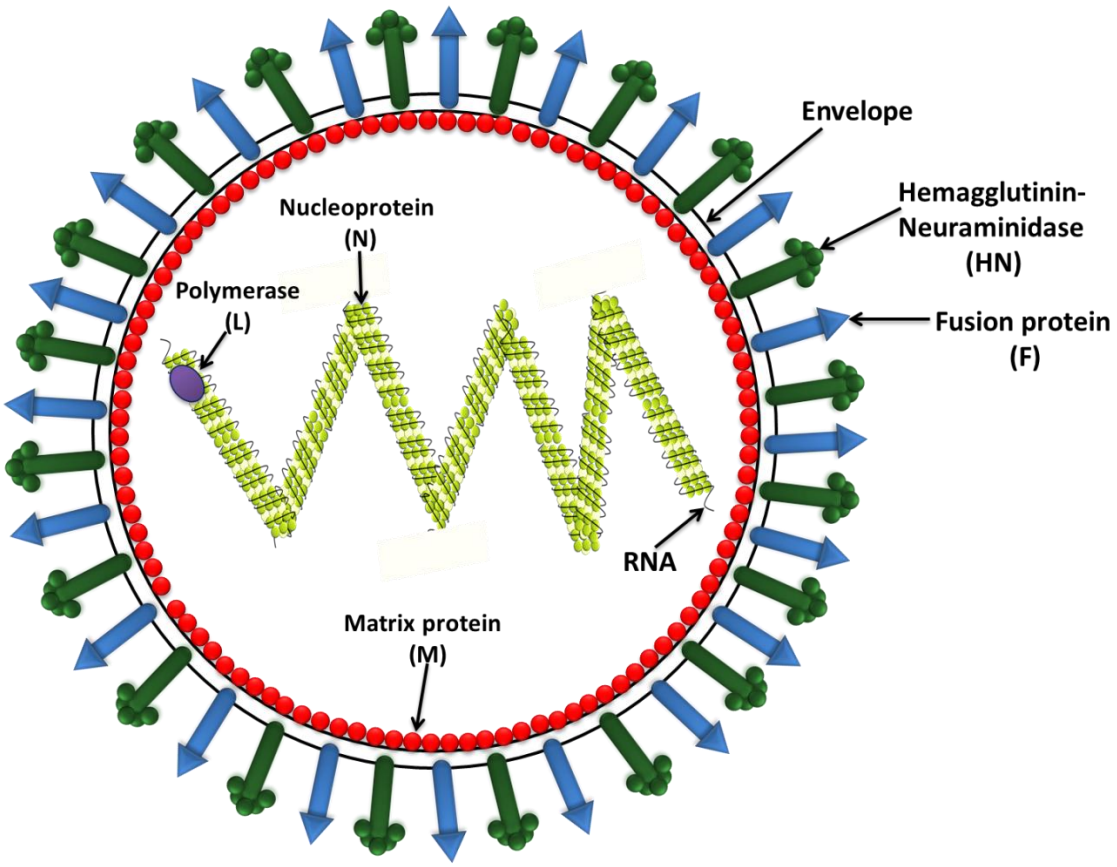

Figure 1.1 Schematic diagram of a typical Newcastle disease virus particle.

Newcastle disease virus (NDV) strains have been divided into three main groups: virulent (Velogenic), moderately virulent (Mesogenic) and non-virulent (Lentogenic), which differ in the number of basic amino acids at the cleavage site of the fusion (F) protein (Brown and Bevins, 2017). Lentogenic strains, especially in adult chickens, may cause minimal or no clinical signs. However, the disease produced by Mesogenic strains may cause mortality that can reach 25% and those by in Velogenic strains may reach up to 100%. High virulent viruses have the ability to replicate in a range of tissues and organs, causing fatal systemic infections (Bilal *et al.*, 2014).

1.3 Genetic variation among Newcastle disease viruses

Like all RNA viruses, genetic change in the Newcastle disease virus can happen. 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. In addition, the different pathogenic virus strains are not identical in their RNA genome sequence (Cho *et al.*, 2007). However, bioinformatics of viral genome from data available in GenBank shows similarities in some regions of M gene between the different virus strains **(Figure 1.2)**.

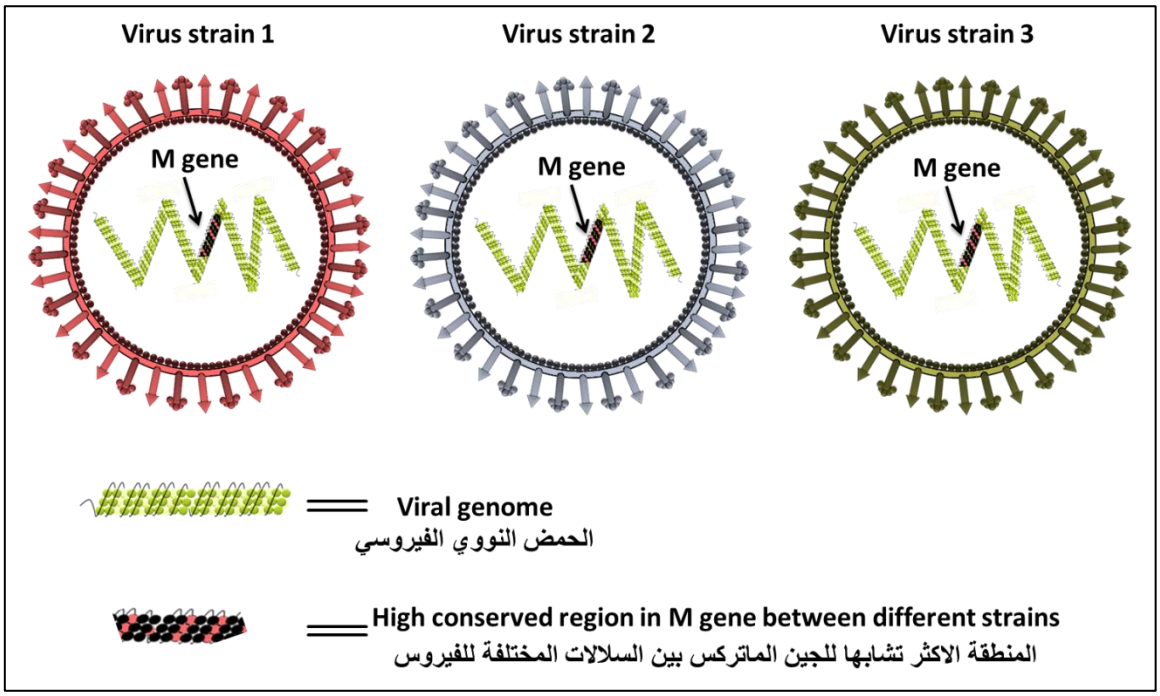

Figure 1.2 The conserved (M) gene of all Newcastle disease virus strains.

1.4 Laboratory diagnosis

Laboratory investigations should confirm the suspected cases and differentiate them from other diseases particularly avian influenza and also some bacterial infections. Techniques for differential diagnosis are essential, as well as the ability to detect mixed infections. Molecular methods such as reverse transcriptase polymerase chain reaction (RT-PCR) have been considered the best identification tool for Newcastle disease virus (Miller and Torchetti, 2014).

However, the genetic variations between virus strains may result in a false-negative diagnosis of NDV infection by using gene specific primers. Therefore, using a developed PCR protocol for NDV detection would be valuable for virus surveillance and screening.

1.5 Aim of the study

The overall aim was to design a pair of universal primers to detect any suspected case with Newcastle disease virus through the amplification of a high conserved gene fragment. The aim was achieved by the following objectives:

1. To determine the conserved regions in the virus genome through the alignment of M genes of different virus strains obtained from GenBank.
2. To determine the location of the forward and reverse primer in the conserved regions using Geneious Inspirational Software for Biologists.
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 conventional and quantitative (Real-Time) RT-PCR techniques on samples collected from chickens and pigeons expected to be infected with Newcastle disease virus.
5. To confirm viral gene amplification by DNA sequencing and bioinformatics.

Chapter Two

Materials and methods

2.1 Materials

Materials, websites and software used throughout the period of this project are as follows:

1. NCBI website
2. Oligoanalyzer 3.1 software
3. Geneious Inspirational Software for Biologists
4. Swabs
5. Centrifuge tubes
6. Eppendorf tubes
7. Viral RNA extraction kit
8. Conventional RT-PCR kit
9. Real-Time RT-PCR kit
10. Universal primers
11. Thermal cycler (for conventional and real-time PCR)
12. Agarose gel
13. Ethidium bromide
14. TBE buffer
15. Gel documentation system
16. Nuclease-free water
17. Phosphate buffer saline
18. Glycerol
19. Microcentrifuge
20. UV hood
21. Micropipettes and different sizes of tips
22. Alcohol, cotton and gloves

2.2 Methods

2.2.1 Design of universal primers

To obtain the complete viral M gene nucleotide sequence, NCBI website (https://www.ncbi.nlm.nih.gov/) was used for this purpose. Highly conserved sequences among several isolates of Newcastle disease viruses of different host species were aligned together to determine the highly conserved regions using Geneious Inspirational Software for Biologists (www.geneious.com) and then used to design the universal primers. The site of the forward and reverse primers was then initially determined by observing the similarities between nucleotides of the selected virus strains **(Figure 2.1)**.

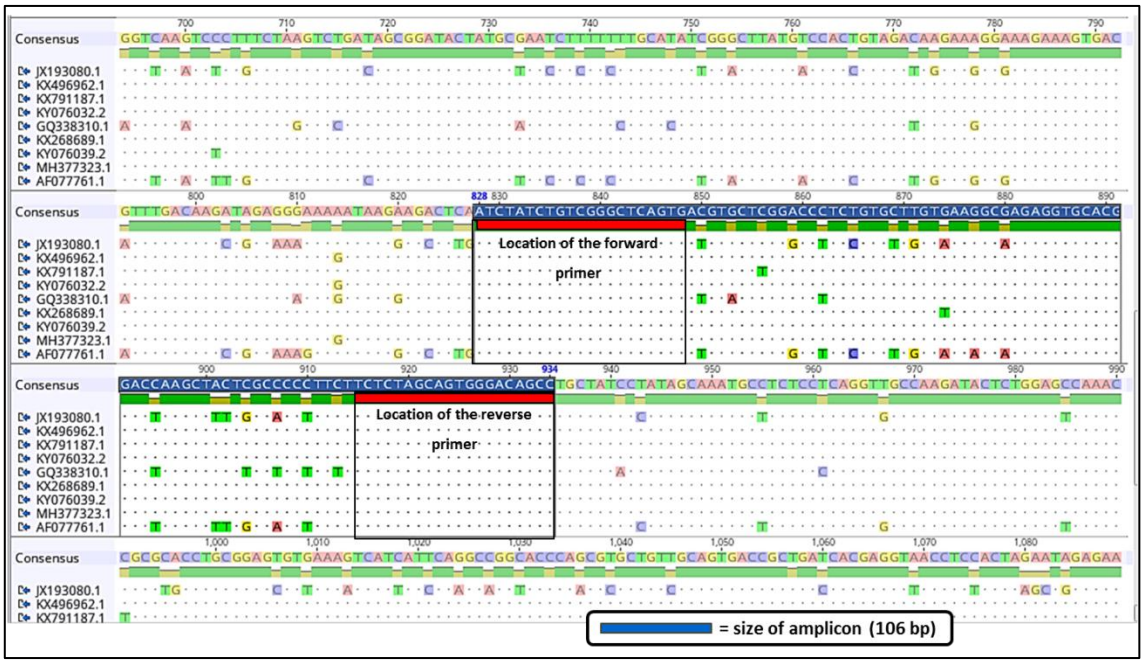

Figure 2.1 Determination of highly conserved nucleotide sequences of Newcastle disease virus M gene. Nucleotide sequences with their accession numbers of partial viral M gene of different virus strains were aligned to determine the conserved areas that were used for designing the forward and reverse primers. The locations of both primers are highlighted in red, and the expected amplicon size (106 bp) is determined by a blue line.

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 and cross dimer formation. Primers were designed to amplify a partial fragment (107 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 Newcastle disease virus M gene.

Gene	Forward primer	Reverse primer	Amplicon size
M	NDV-F-828-848 (21 bp) ATCTATCTGTCGGGCTCAGTG	NDV-R-934-915(20 bp) GGCTGTCCCACTGCTAGAGA	107 bp

2.2.2 Sample collection

A total of 20 nasopharyngeal swabs were collected from 10 chickens, and 10 pigeons expected to be infected with Newcastle disease virus in Basrah governorate **(Figure 2.2)**. Each sample was collected in a sterile phosphate buffer saline (PBS) with glycerol (1:1). 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)**.

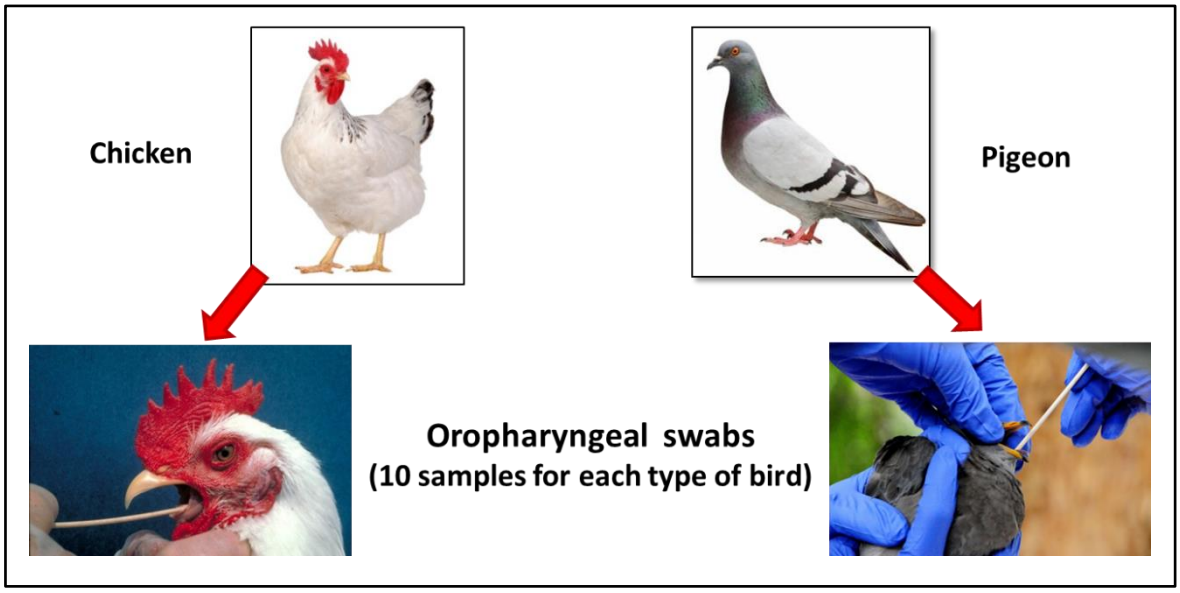

Figure 2.2 Method of sample collection from chicken and pigeon.

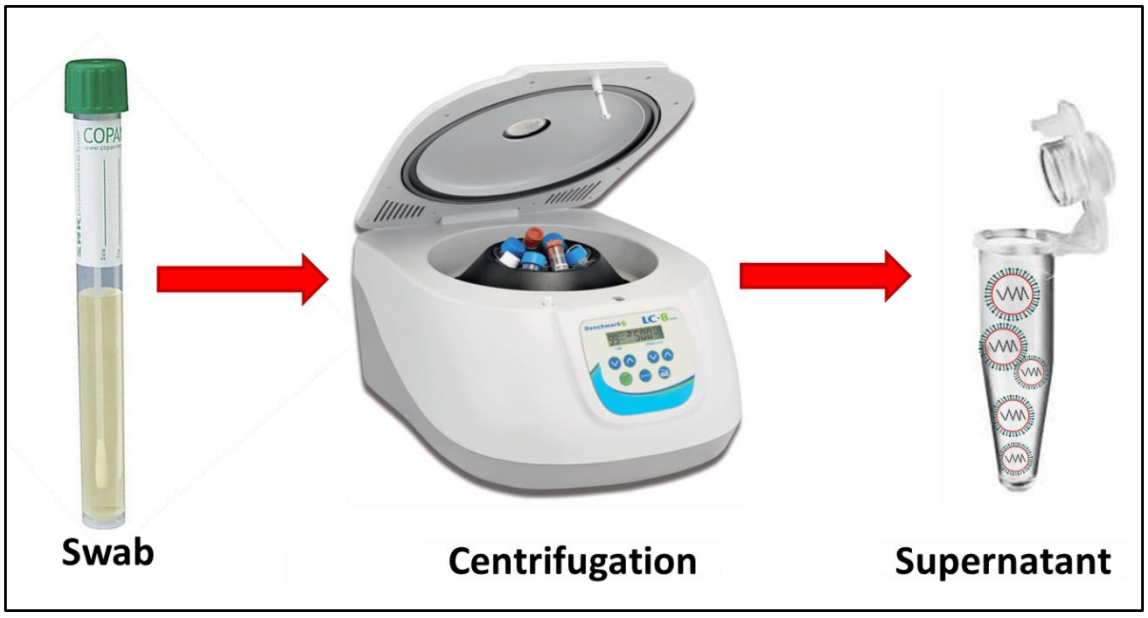

Figure 2.3 Clarification of nasopharyngeal swabs. All swabs were clarified by centrifugation and the supernatants were then collected.

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)**.

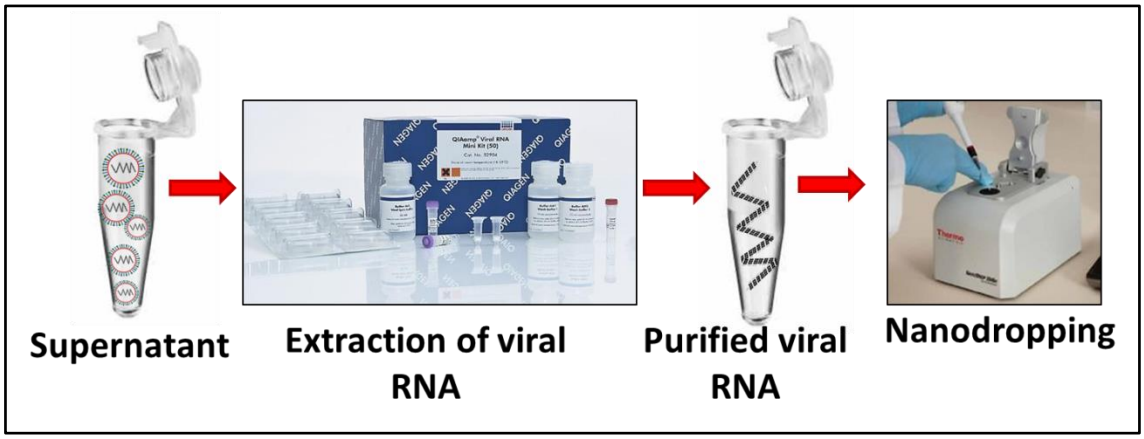

Figure 2.4 Viral RNA extraction and quantification. Viral RNA was extracted from supernatants and quantified by NanoDrop spectrophotometer.

2.2.4 Conventional reverse transcriptase polymerase chain reaction (RT-PCR)

Amplification of a partial region of Newcastle disease virus M gene was performed using One Step RT–PCR kit (Bioneer). The designed forward primer 5'- ATCTATCTGTCGGGCTCAGTG -3' and reverse primer 5'- GGCTGTCCCACTGCTAGAGA -3' were used to amplify 107 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. Two negative control samples (without RNA template, or without primers) were prepared in each PCR run along with two positive control which were represented by using viral RNA extracted form a live virus (LaSota) vaccine. 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 Newcastle disease virus M gene.

Step	Temperature	Time
cDNA synthesis	45˚C	30 minutes
Initial denaturation	95˚C	2 minutes
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.

2.2.6 DNA sequencing and sequence analysis

Ten microliters of the PCR product along with 17 picomole per microliter of forward and reverse primers that were used to amplify the viral M gene were sent to Macrogen company/ South Korea for sequencing. The sequences were edited, assembled and aligned by using Geneious Inspirational Software for Biologists (www.geneious.com).

2.2.7 Real-Time Reverse transcriptase polymerase chain reaction (rRT-PCR)

Amplification of a partial region of Newcastle disease virus M gene was also performed using One Step Bright Green qRT–PCR Low Rox kit (abm). The designed forward primer 5'- ATCTATCTGTCGGGCTCAGTG -3' and reverse primer 5'- GGCTGTCCCACTGCTAGAGA -3' were used to amplify 107 base pare (bp) fragment. Both cDNA synthesis and PCR amplification were performed in a single tube using this system. The RT-PCR conditions were: cDNA synthesis at 42˚C for 15 min, initial denaturation at 95˚C for 10 min followed by 40 cycles of: denaturation at 95˚C for 15 s, annealing at 58˚C for 60 s **(Table 2.3)**.

Table 2.3 Real-Time RT-PCR conditions for amplifying a partial fragment of Newcastle disease virus M gene.

Step	Temperature	Time
cDNA synthesis	42˚C	15 minutes
Initial denaturation	95˚C	10 minutes
40 cycles of: Denaturation Annealing	 95˚C 58˚C	 15 seconds 60 seconds

Chapter Three

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)**.

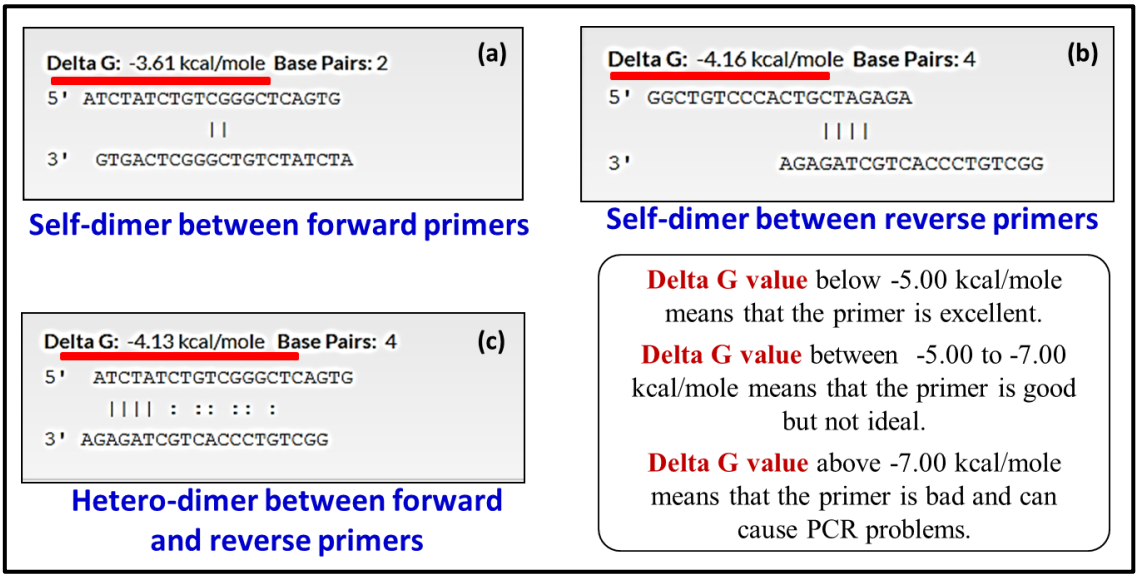

Figure 3.1 Determination of universal primer efficiency. Delta G values of self-dimer between forward primers (a), reverse primers (b), and hetero-dimer between forward and reverse primers (c) were less than -5 which means that all primers are excellent and would not cause PCR problems.

3.2 Detection of Newcastle disease viruses by conventional RT-PCR

Using the designed universal primers, PCR products of partial matrix (M) gene of Newcastle disease virus were separated on 1.5% agarose gel pre-stained with ethidium bromide. Nineteen samples (9 chickens, and 10 pigeons) out of 20 gave positive results on agarose gel. The separation of selected PCR products on agarose gel is shown in **Figure 3.2**.

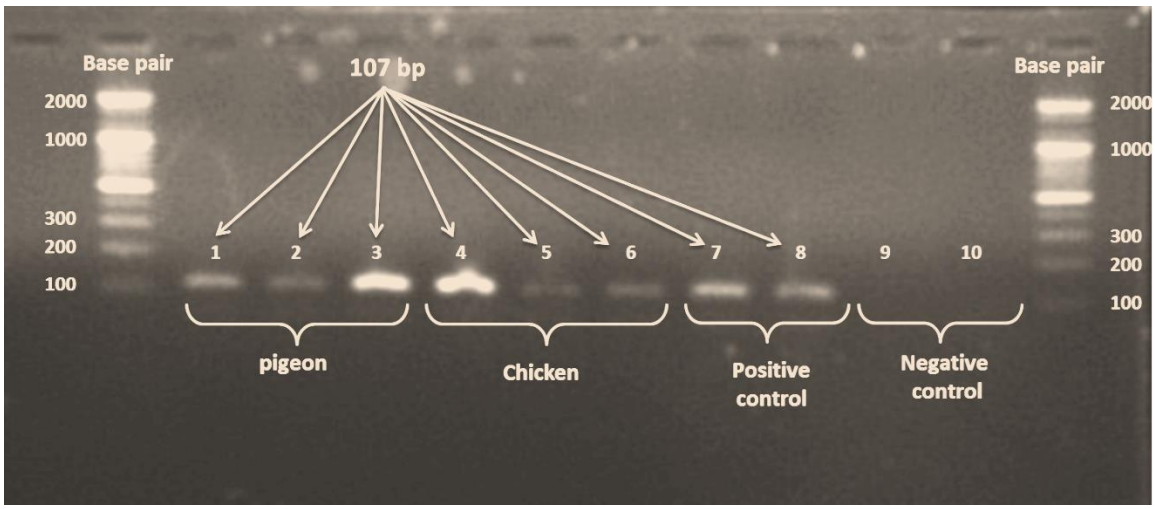

Figure 3.2: PCR product of partial matrix (M) gene of Newcastle disease virus on 2% agarose gel stained with ethidium bromide.

The results showed the amplification of 107 bp from oropharyngeal specimens collected from pigeons (lane 1-3), chickens (lane 4-6), and positive control (lane 7 and 8), respectively. Negative controls (lane 9 and 10) which represent reaction without RNA or primers, respectively did not show any evidence of amplification.

3.3 DNA sequencing and sequence analysis

The PCR product was successfully sequenced. DNA sequencing results were edited using Geneious Inspirational Software for Biologists. Clear DNA traces (without secondary structure) were easily noticed in both forward and reverse sides of the nucleotide sequences **(Figure 3.3)**.

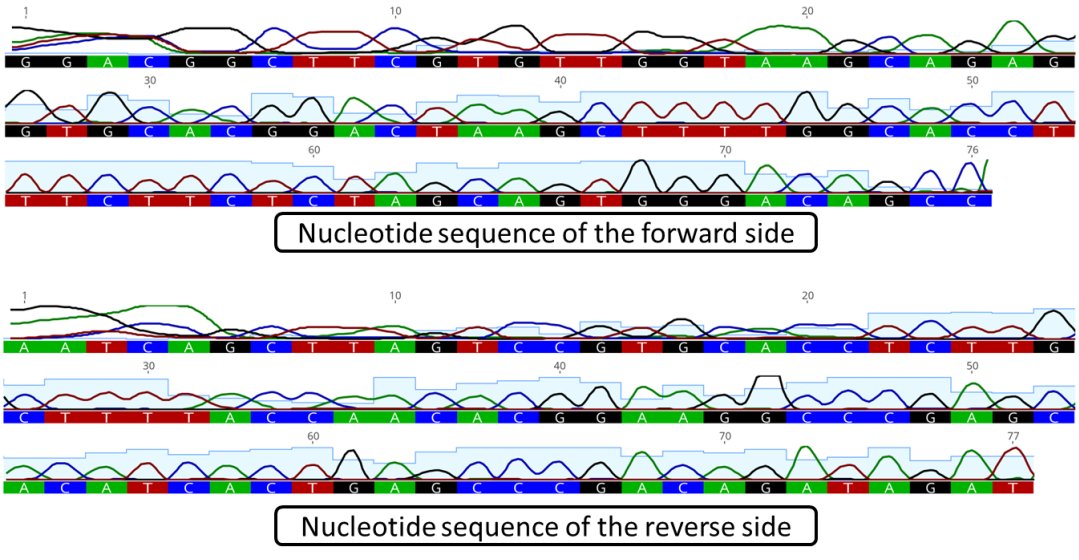

Figure 3.3: DNA sequencing of partial matrix (M) gene of Newcastle disease virus.

The forward and reverse side of DNA sequences were then assembled (merged by overlapping) by using the same software to generate the 107 nucleotides sequence which is located between the forward and reverse priming sites **(Figure 3.4)**.

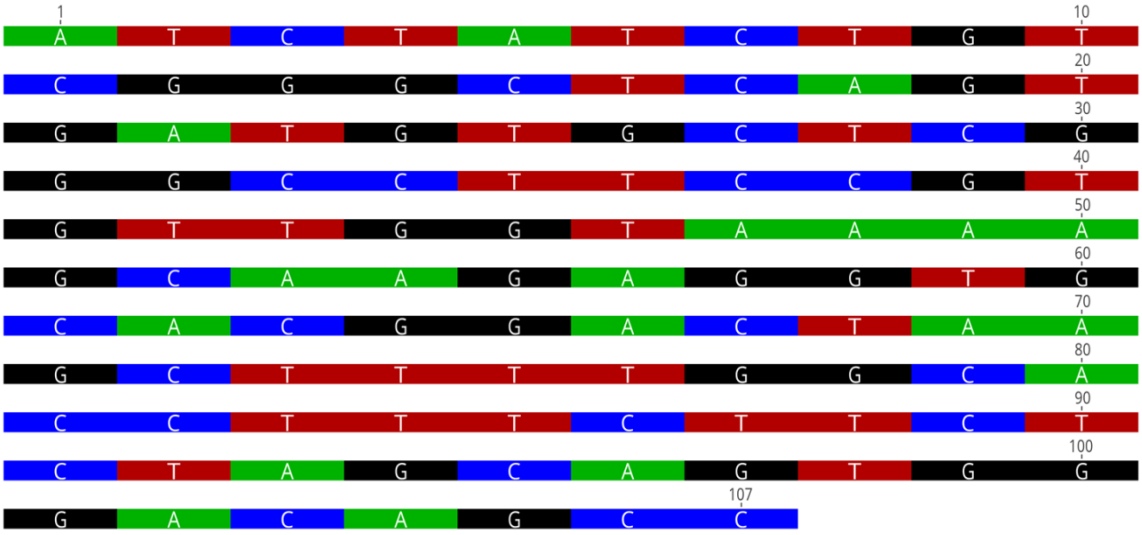

Figure 3.4: Final DNA sequence of partial matrix (M) gene (107 bp) of Newcastle disease virus. Forward priming site (ATCTATCTGTCGGGCTCAGTG) is located between nucleotide number 1 to 21, while the reverse priming site (GGCTGTCCCACTGCTAGAGA) is located between nucleotide number 107 to 88). The length of the assembled sequence is 107 bp, which is comparable with the size of the PCR product seen on agarose gel.

The final DNA sequence was then aligned with sequences available in GenBank. The results of gene alignment confirmed the infection with the Newcastle disease virus **(Figure 3.5).**

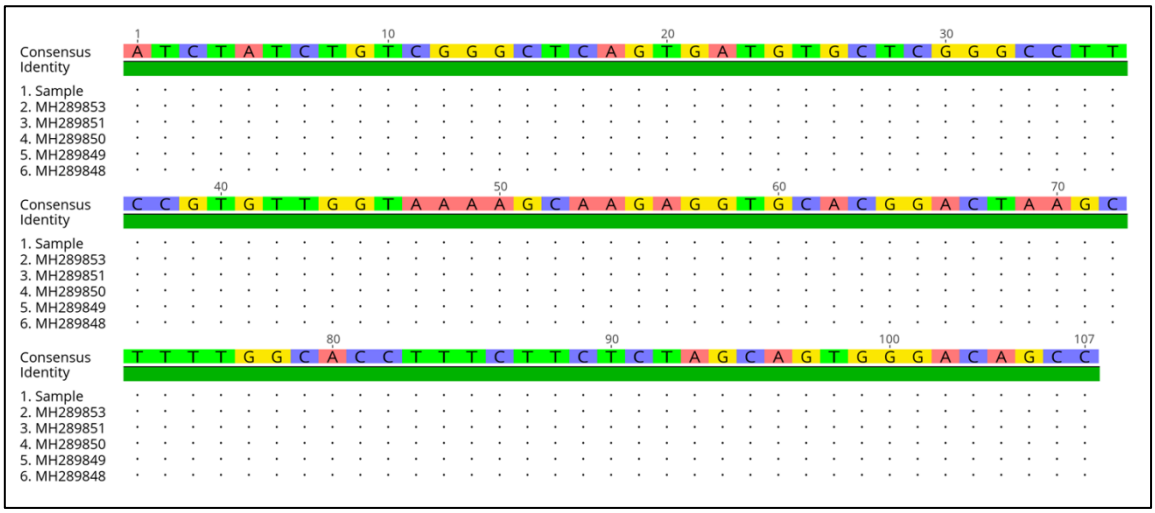

Figure 3.5 Partial M gene alignment between the amplified viral gene fragment and genes available on GenBank. Identical sequences between the tested sample (1) and the online sequences of Newcastle disease virus with their accession numbers (2 to 6) were observed. This result confirmed the infection of the birds with the Newcastle disease virus.

3.4 Detection of Newcastle disease viruses by Real-Time RT-PCR

Using the designed universal primers, the partial matrix (M) gene of Newcastle disease virus was also amplified successfully and detected by using Real-Time RT-PCR through the reaction of fluorescent molecules with the amplified DNA to produce a fluorescent signal **(Figure 3.6)**. These findings confirm that the these primers can be used in the future to detect any suspected case with Newcastle disease virus and to determine viral load in samples, which reflects the intensity of infection.

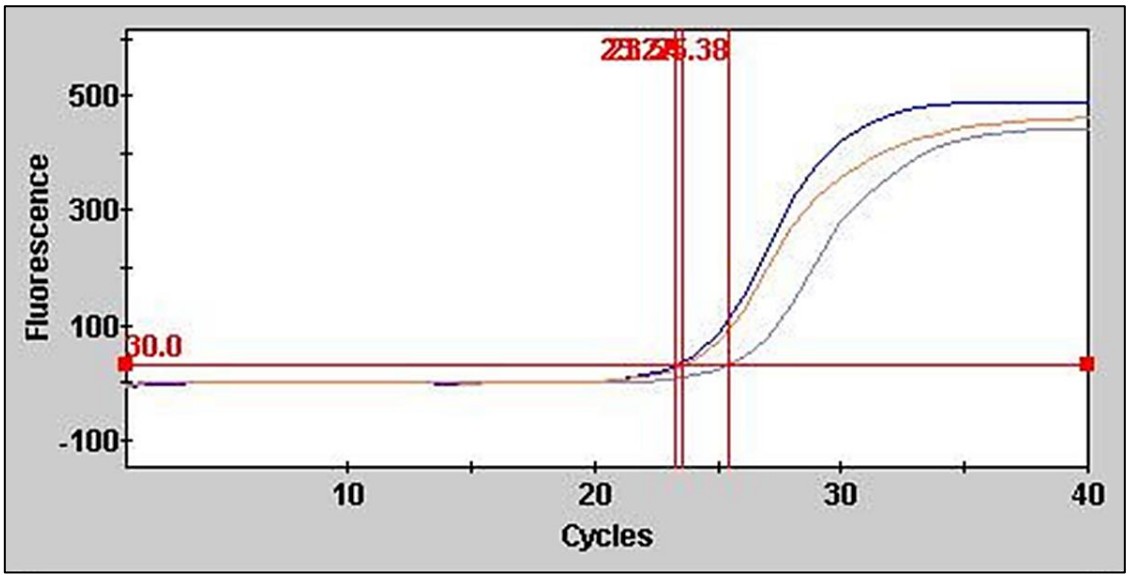

Figure 3.6: Detection of partial matrix (M) gene of Newcastle disease virus by Real-Time RT-PCR. Positive results were observed on samples taken from chickens, pigeons along with the positive control (live virus vaccine).

Chapter Four

Discussion

Discussion

Newcastle disease virus has been classified into different pathogenic strains according to the variations in the fusion (F) proteins. There are several pathogenic strains have been circulating in different bird species (Heiden *et al.*, 2014). The sensitivity and specificity of PCR-based methods for the detection of virus strain are most critically determined by the choice of primer sequences.

Viral genome amplification techniques based on RT-PCR assays are regarded as a specific diagnosis to confirm Newcastle disease virus infection (Yi and Liu, 2011). The sequences of the primer sets that are routinely used for PCR-based detection of Newcastle disease virus may be appropriate for the detection of specific virus strains circulating in birds. 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 strain.

In this study, an extensive amount of the sequence information available for Newcastle disease virus was used to design a pair of universal primer for the diagnostic purposes. The designed forward and reverse primers span conserved sequences of a fragment in M gene of Newcastle disease virus and have no homology to nucleotide sequences from other species available in NCBI database. The experimental data gained in this study confirmed that PCR amplification with this pair of primers does not have any problems. This was also confirmed by sample sequencing and sequence analysis which gave an excellent nucleotide sequence data. Obtaining a good sequencing result without secondary structure or trace overlapping is entirely due to the correct PCR conditions and the good quality of primers used in gene amplification. 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). More importantly, this pair of primer has also given excellent results of virus detection using quantitative (Real-Time) RT-PCR technique. This optimization will be useful for virus quantification and giving a clear picture of the severity of the disease and viral load. Therefore, it is strongly recommended to use the designed pair of primers for virus detection and/or quantification by performing conventional and Real-Time RT-PCR techniques, respectively.

In conclusion, by performing this novel PCR-based assay, diagnosis of Newcastle disease with any virus strain in different host species 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 the disease.

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الخلاصة

تحتوي فيروسات مرض نيوكاسل على الحمض النووي الرايبوزي (RNA) والذي يكون بشكل غير مجزأ الى عدد من القطع. تنقسم هذه الفيروسات إلى سلالات مختلفة اعتمادا على درجة الامراضية للفيروس. ان الحمض النووي الفيروسي ليس متطابقًا بين هذه السلالات ، وبالتالي يتطلب التشخيص الجزيئي استخدام زوج محدد من البريمرات لتضخيم منطقة معينة في الحمض النووي لكل سلالة. كان الهدف من هذه الدراسة هو ابتكار طريقة موثوقة وبسيطة لتشخيص أي إصابة بفيروس مرض النيوكاسل في تفاعل أنزيمي واحد باستخدام زوج جديد من البريمرات الشاملة. لتصميم هذه البريمرات, تم تحديد مناطق ذات تشابه كبير من جين الماتريكس (M) بين مجموعة من سلالات مختلفة من فيروس مرض النيوكاسل والتي تتوفر في قاعدة بيانات NCBI. 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 20 مسحة فموية بلعومية من 10 حمامات و 10 دجاجات تظهر عليها علامات مرض نيوكاسل. تمت تمرير جميع العينات لاستخراج الحمض النووي الرايبوزي وتضخيم الجينات باستخدام تفاعل البلمرة المتسلسل التقليدي (RT-PCR) والكمي الحقيقي (Real-Time RT-PCR) وكذلك تحديد تسلسل القواعد النتروجينية للقطعة التي تم تضخيمها. أظهرت النتائج أن كلا البرايمران (الامامي والعكسي) كانت ذات جودة 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 و Real-Time RT-PCR أن قطعة من الجين M تم تضخيمها بنجاح من معظم العينات التي تم جمعها من الحمام والدجاج وهذا ما تم تأكيده أيضًا في تطبيق تقنية تسلسل القواعد النتروجينية والمعلوماتية الحيوية. تؤكد هذه النتائج أن هذا الزوج الجديد من البريمرات الشاملة يمكن استخدامه للكشف عن أي فيروس لمرض نيوكاسل ومن اي مضيف في تفاعل إنزيمي واحد.

تصميم بريمرات شاملة للكشف عن فيروسات مرض النيوكاسل في تفاعل انزيمي واحد

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