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Identification and Phylogenetic Analysis of Newcastle Disease Virus in the West Bank, Palestine

By

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by

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Identification and Phylogenetic Analysis of Newcastle Disease Virus in the West Bank, Palestine

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Abstract

Newcastle disease (ND) is one of the most economically devastating diseases to affect the poultry industry world-wide and is listed as a notifiable disease by the OIE. Live vaccines based on low-pathogenic strains of genotype II are routinely used to prevent ND, but improper vaccination may cause farm outbreaks and it is important to distinguish vaccine-related infections from highly pathogenic (velogenic) strains of other genotypes. The F-gene has been used to classify Newcastle Disease Virus (NDV) into 21 major genotypes, and the F protein cleavage site sequence at amino acids 112-117 is a well-characterized determinant of NDV pathogenicity.

This study is the first to infer pathogenicity and genotype for NDV in the West Bank. Swab (years 2021/22) and previously purified RNA (year 2018) samples were obtained from both vaccinated and unvaccinated birds showing symptoms of Newcastle disease in the West Bank between 2018 and 2022, and those positive for NDV were sequenced using a 362nt amplicon of part of the F-gene spanning the F-protein cleavage site sequence. In addition, NDV was isolated from a locally available vaccine and sequenced for comparison with the field isolates. Pathogenicity inference and genotyping based on a maximum likelihood phylogeny was performed after empirical validation that the amplicon sequence used would recapitulate reference genotypes previously defined by full-length sequencing.

NDV was confirmed from four outbreaks in Hebron during 2021/22, one from Bethlehem in 2021; and four from Tubas, Nablus, and Jerusalem in 2018. Complete and partial F-gene reference sequences were aligned separately and these produced trees with congruent topology at the genotype level. Addition of West Bank sequences to the reference sequences revealed that five West Bank isolates were of genotype VII and these could be distinguished into two sub-types that have existed since the earliest samples from 2018. Two of the isolates clustered with sub-group VII.1.1, while the remaining three clustered with sub-group VII.2. These isolates all had an inferred

velogenic F-protein cleavage site. The remaining four isolates in this study were assigned to genotype II and were identical to a sequence that came from a locally available vaccine.

It is concluded that in the West Bank symptomatic ND is commonly caused by vaccination, and that velogenic strains of NDV are circulating amongst poultry. The presence of highly pathogenic virulent strains along with vaccine-induced disease clearly demonstrates a need for the placement of NDV surveillance procedures as well as awareness campaigns for commercial and backyard farmers about ND prevention, vaccination, and other health measures to manage NDV.

تحديد فيروس نيوكاسل وتحليل النشأة والعلاقات التطورية للفيروس في الضفة الغربية، فلسطين

ملخص الدراسة:

يعدّ مرض نيوكاسل أحد الأمراض التي تصيب الدواجن في مختلف أنحاء العالم، وهو من الأمراض التي تؤثر بصورة سلبية كبيرة في الناحية الاقتصادية، كما أُدرج كمرض يجب الإبلاغ عنه من قِبَل المنظمة العالمية لصحة الحيوان. (OIE) ويُشار إلى أنه يتم استخدام اللقاحات الحية المحتوية على سلالات ضعيفة النمط الجيني الثاني بهدف الوقاية من الإصابة بمرض نيوكاسل، إلا أنّ استخدام اللقاحات غير المناسبة يؤدي إلى تفشي هذا المرض في مزارع الدواجن. ولا بد من التنبيه إلى ضرورة التمييز بين العدوى التي تنتج عن استخدام اللقاحات، والعدوى التي تنتج عن سلالات مرضية شديدة العدوى لأنماط جينية أخرى، وقد تم استخدام الجين F من أجل تصنيف فيروس نيوكاسل إلى 21 نمطًا وراثيًا رئيسيًا، وكان تسلسل الأحماض الأمينية 112 - 117 في موقع انقسام البروتين F مُحدِّدًا مناسبًا للآلية المرضية لمرض نيوكاسل.

وتعد هذه الدراسة الأولى من نوعها في الضفة الغربية التي تتناول الآلية المرضية والنمط الجيني لإصابة الدواجن بفيروس نيوكاسل. بالاعتماد على دراسة التركيب الجيني لفيروس هذا المرض باستخدام عينات مسحية (في عام 2022/2021)، وعينات من الحمض النووي المنقّى سابقًا (في عام 2018) من الدواجن المُلقّحة وغير المُلقّحة في الضفة الغربية، والتي تظهر عليها أعراض الإصابة بمرض نيوكاسل بين عامي 2018 - 2022، وتم ترتيب العينات الإيجابية لفيروس مرض نيوكاسل باستخدام مضخم 326 نيوكليوتيد من جزء من الجين F الذي يمتد في تسلسل موقع انقسام البروتين F. كما تم عزل فيروس نيوكاسل من لقاح متوفر على المستوى المحلي ومعرفة تسلسله لمقارنته مع العزلات الحقلية. وكان الاستدلال على الآلية المرضية والنمط الجيني بالاعتماد على أقصى احتمالية لتطور السلالات بعد التحقق التجريبي من أنّ جزء الحمض النووي المستخدم سيوضح الأنماط الجينية المرجعية التي تم تحديدها سابقًا بالتسلسل كامل الطول.

تم تأكيد وجود أربع إصابات بين الدواجن بفيروس نيوكاسل في مدينة الخليل في عام 2022/2021، وإصابة واحدة في مدينة بيت لحم في عام 2021، بالإضافة إلى أربع إصابات في طوباس ونابلس والقدس في عام 2018. وجرى ترتيب التسلسلات المرجعية الجزئية والكاملة للجين F كل على حدة، وكانت النتيجة أشجارًا ذات طوبولوجيا متطابقة النمط الجيني. إنّ إضافة التسلسلات الضفة الغربية إلى التسلسلات المرجعية كشفت أنّ خمسة تسلسلات معزولة في الضفة الغربية كانت من النمط الجيني السابع، ويمكن تقسيمها إلى نوعين فرعيين كانا موجودين في العينات الأولى التي أخذت في عام 2018، وهما كالآتي: تسلسلان اثنان ينتميان إلى المجموعة الفرعية (VII 1.1)، وثلاثة تسلسلات تنتمي إلى المجموعة الفرعية (VII.2). وكان لجميع هذه التسلسلات المعزولة موقع انقسام بروتين F عالي الإمراض، وتم تعيين العزلات الأربعة المتبقية في هذه الدراسة إلى النمط الجيني الثاني، وكانت مطابقة للتسلسل الناتج عن اللقاح المستخدم على المستوى المحلي.

خلُصت الدراسة إلى أن أعراض الإصابة بفيروس نيوكاسل في الضفة الغربية ظهرت في الغالب نتيجة استخدام اللقاحات، وأنّ سلالات هذا الفيروس منتشرة بين الدواجن، ويدل وجود سلالات مسببة للمرض بالإضافة إلى الإصابة الناتجة عن استخدام اللقاحات على ضرورة وضع إجراءات لمراقبة فيروس نيوكاسل، والبدء بحملات توعوية للمزارعين حول كيفية وقاية الدواجن من الإصابة بالمرض، إلى جانب اتخاذ التدابير الصحية الأخرى التي من شأنها السيطرة على فيروس نيوكاسل.

DECLARATION

I declare that the master thesis entitled "**Identification and Phylogenetic Analysis of Newcastle Disease Virus in the West Bank, Palestine**" is my own original work, and thereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name: Rana Walid Jamil Seder

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List of abbreviations

BLAST	Basic local alignment search tool
Bp	Base pair
cDNA	Complementary deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
F gene	Fusion protein
F	Phenylalanine
FPV	Fowlpox virus
G	Glycine
GTR	General time reversible model
HF	Buffer solution optimized for high-fidelity DNA
HI	Hemagglutination-inhibiting
HN	Hemagglutinin-neuraminidase
HVT	Herpesvirus
K	Lycine
L	large RNA polymerase protein
M	matrix protein
MEGA	Molecular Evolution Genetic Analysis
ML	Maximum likelihood
ml	Milliliter
mM	Millimolar
µM	Micromolar
µl	Microliter
MSA	Multiple sequence alignment
NCBI	National Center for Biotechnology Information
ND	Newcastle disease
NDV	Newcastle disease virus
nt	nucleotide
NTC	No template control
OIE	Office International des Epizooties\ World Organization for Animal Health
P	Phosphoprotein
PAL	Palestine
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
Q	Glutamine
R	Arginine

RFLP	Restriction enzyme fragment length
RNA	Ribonucleic acid
SIAS	Sequences Identities and Similarities
SPF	Specific pathogen-free
SSIV	Buffer use with SuperScript™ Invitrogen Reverse Transcriptase
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tm	Melting temperature
UV	Ultraviolet
VIR	Virulence interferon resistance
w/v	Weight to volume

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1. CHAPTER ONE

1. Introduction

1.1 Newcastle Disease

Newcastle disease is an avian viral disease that is considered one of the most economically devastating diseases affecting the poultry industry. It causes a high rate of morbidity and mortality in susceptible flocks, resulting in significant financial losses, as it can cause a decrease in egg production and egg quality (Igwe, Ihedioha and Okoye, 2018), as well as a loss in weight (Sedeik *et al.*, 2019). The disease is highly contagious, and can spread through an entire flock within two to six days. Moreover, entire flocks can be exterminated, if not by the disease itself then by mandatory culling carried out as a control measure.

Affected birds become anorexic, and suffer from green diarrhea, and nasal discharge with difficulty breathing, and within days start to display neurological symptoms such as paralysis of the legs and wings, blindness, incoordination, characteristic twisting on the neck to one side (torticollis), and moving continuously in a circle. Autopsy most often reveals lesions affecting the gastrointestinal, nervous, respiratory, and reproductive systems. Clinical signs vary greatly depending on factors such as virus strain, infected bird species, host age (young birds are the most susceptible), concurrent infection with other pathogens, and immunological condition. Infection with highly virulent virus strains can cause large numbers of birds to die with few clinical indications in some cases (Hines and Miller, 2012). In addition to chickens, pigeons, cormorants, psittacines, pheasants, peafowl, as well as more than 200 other avian species have been reported to be affected, and it is likely that all birds are susceptible to this disease (Cardenas Garcia *et al.*, 2013).

This disease was first documented in 1926 in Java, Indonesia, and then in Newcastle-upon-Tyne, England the following year (hence the name (Alexander, 2001). The disease could possibly be responsible for previous incidents, such as when all domestic fowl were wiped out from an unknown disease in northwest Scotland in 1898. ND is transmitted by direct contact with diseased birds, or by contact with their feces or other

discharges or contaminated objects or surfaces, where the causative virus can survive for several weeks in the environment in contaminated material (Roberts, Souillard and Bertin, 2011). Complicating matters, migratory wild birds have been found to carry the disease, and may be responsible for spreading the disease over entire continents (Karamendin and Kydyrmanov, 2021).

Several incidents in the past decade illustrate the devastating damage that ND can cause, which also threatens the food supply in fragile economies in addition to economic losses. In the Punjab province of Pakistan alone from 2011 to 2012, NDV killed 45 million broiler chickens, resulting in an economic loss of 6 billion PKR. Also, in Pakistan in 2012, an NDV outbreak in the Jallo wildlife Park resulted in the death of 190 peacocks in the span of one week (Munir *et al.*, 2012). On average, 60 countries around the world reported outbreaks of ND from 2013 to 2015. Significantly, Israel experienced an epidemic of ND that peaked in 2011-2012 and affected 393 flocks (Wiseman, Berman and Klement, 2018).

1.2 Newcastle Disease Virus Structure and Genome Organization

Newcastle disease is caused by the Newcastle disease virus (NDV), also known as *Avian paramyxovirus type-1*. It is of the genus *Avulvavirus* and family *Paramyxoviridae* (De Leeuw and Peeters, 1999), (Amarasinghe *et al.*, 2019). This virus is an enveloped virus whose nucleic acid is non-segmented, negative sense single stranded RNA of 15,186 to 15,198 nucleotides in length (Cai *et al.*, 2011). The genome contains eight genes (**Fig 1.1**). Six of these genes code for structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), and large RNA polymerase (L) protein, which are found 3' to 5' in the order mentioned (Murulitharan *et al.*, 2013). The other two genes are for non-structural proteins, and are expressed by editing of the P protein mRNA transcription (Duan *et al.*, 2020).

1.3 Pathogenicity of NDV

Based on pathogenicity indices (mainly mean death time in nine to ten-day old embryonated chicken eggs and intracerebral pathogenicity index in one day old chickens), NDV strains are classified into three pathotypes: velogenic, mesogenic, and lentogenic. Velogenic strains can be either neurotrophic or viscerotrophic, and cause the most severe

form of the disease. Affected birds may suffer from hemorrhagic gastroenteritis, pneumonia, and/or encephalitis. Mesogenic strains cause more moderate respiratory and neurological symptoms, while lentogenic strains demonstrate low virulence, and may cause only mild symptoms (Piacenti *et al.*, 2006), (Moura *et al.*, 2016).

Interestingly, the major determining factor that differentiates between the NDV pathotypes is the amino acid composition of the cleavage site of the fusion (F) protein, specifically at positions 112-117. The F protein is responsible for the fusion of the viral envelope with the host-cell membrane. In order to carry out this function, the F protein must be activated by proteolytic cleavage of the F0 precursor to produce F1 and F2 subunits that are linked by a disulfide bond (Wang *et al.*, 2017). The amino acid composition of this cleavage site determines which kind of cellular proteases are able to cleave the F protein precursor. Virulent NDV strains contain polybasic amino acids at the cleavage site, and a phenyl alanine residue at position 117, which are recognized and cleaved by ubiquitous intracellular subtilisin-like proteases such as furin, PC6 and PACE 4 that are found in most host cells (Panda *et al.*, 2004). The intracellular protease efficiently cleaves the F protein in most of the host tissues, so the virus is spread systemically, thus resulting in a severe form of the disease. On the other hand, avirulent NDV strains have fewer basic residues, and usually a leucine residue at position 117, making the cleavage site insensitive to intracellular proteases, and the cleavage of the F protein is restricted to the extracellular respiratory and enteric tracts, where it is cleaved by trypsin-like enzymes, resulting in a considerably more mild form of the disease. Studies have shown that velogenic strains require an arginine at position 113, arginine or lysine at position 115, arginine at position 116, and phenylalanine at position 117 as shown in (**Fig. 1.1**) (Collins, Bashiruddin and Alexander, 1993). Accordingly, the amino acid composition of the F protein cleavage site can be used for rapid and simple pathotyping of NDV strains, and in fact the OIE refers to the amino acid chemistry of the cleavage site in its definition of virulent NDV strains, specifying that virulent strains have an intracerebral pathogenicity index of 0.7 or higher and a cleavage site with multiple basic amino acids and phenylalanine at position 117 (Afonso *et al.*, 2012).

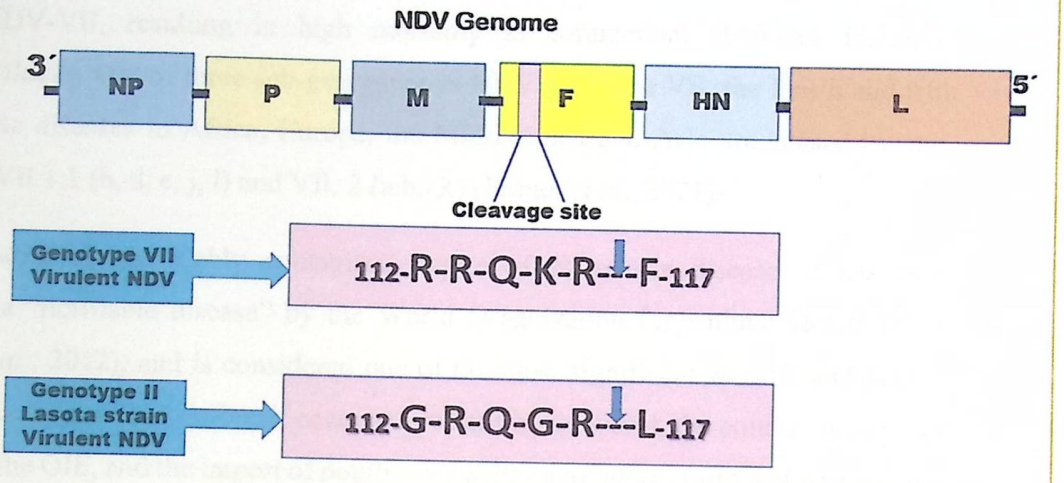


Figure 1.1: The proteolytic cleavage site of ^{112}R-R-Q-K-R ↓F ^{117} is characteristic for velogenic NDV in class II, genotype VII, and ^{112}G-R-Q-G-R ↓L ^{117}is characteristic for lentogenic NDV in class II, genotype II. (R- arginine; K- lysine; Q- glutamine; G- glycine; F- phenylalanine; L- Leucin; Arrow: cleavage position; Number: residue position in F protein).

1.4 Epidemiology of Newcastle Disease

Following the outbreaks documented in 1926 and 1927, the disease slowly spread and resulted in a global pandemic over the next two decades. A second more rapid pandemic also occurred in the 1960s, which spread over the entire globe in a matter of only four years, facilitated probably by the commercialization of poultry production and the exchange of exotic birds. The unlawful import at that time of species of pet birds was a major source of the highly virulent NDV virus, which played a major role in the spread of the virus in commercial poultry around the world (PERDUE and SEAL, 2000). A third pandemic occurred in the 1980s, which began in racing pigeons and eventually affected the poultry sector, causing significant economic damage (Lumeij and Stam, 1985). Racing, exhibitions and trading accelerated the spread of the virus to different regions of the world (Kaleta, Alexander and Russell, 2008). The fourth pandemic, which began in Southeast Asia around the mid-1980s and has since expanded to the Middle East, Europe, America, and Africa (Bello *et al.*, 2018). Various genotypes have been implicated with various ND

panzootic. Most recent ND outbreaks in Southeast Asia have been caused by highly virulent NDV-VII, resulting in high mortality in commercial chickens, including vaccinated flocks. Out of three sub-genotypes in NDV genotype VII, the fourth and fifth ND zoonotic diseases in Africa, Europe, the Middle East and Asia are caused by sub-genotypes VII.1.1 (b, d, e, j, l) and VII. 2 (a,h,i,k) (Rabiei *et al.*, 2021).

Because of the highly contagious nature of Newcastle disease, it has been designated a "notifiable disease" by the World Organization for Animal Health (OIE) (Afonso *et al.*, 2012), and is considered one of the most significant wildlife and poultry diseases. If a virulent ND outbreak occurs anywhere in the world, the country is required to report to the OIE, and the import of poultry or poultry derivatives from that country may be suspended. While Western Europe, the United States, and Brazil have had great success in reducing incidence of the disease, many mainly developing countries in Latin America, Eastern Europe, Africa, the Middle East, and Asia still struggle to keep the disease under control, and are subject to regular burdens due to outbreaks. The prevalence of backyard poultry, small scale farming, and traditional live bird markets in these regions contribute to the spreading of the ND virus, and these operations are often disconnected from standard government issued vaccination and biosecurity protocols against the disease.

1.5 Newcastle Disease Virus Classification Schemes and Phylogenetics

NDV exists as a single serotype, but its genetic diversity is vast. This may be due to the broad circulation of NDV, as well as the inherently high error rate of its viral RNA polymerase. Since the discovery of NDV, many techniques have been used to classify the different strains. Early techniques relied on traits (biological properties of virus) such as pathogenicity, plaque formation, thermostability, analysis of polypeptides, and hemagglutination inhibition patterns. Later gene-based techniques were used to classify NDV strains. Restriction fragment length polymorphism (RFLP) analysis of a region of the F gene was used by Ballagi-Pordány *et al* (1996) to group strains, but RFLP is notoriously irreproducible. With the development of rapid sequencing technologies, the nucleotide sequences of particular NDV genes were used for classification and phylogenetics, which attempts to infer the evolutionary relationship between virus strains.

Phylogenetic analysis can be used in studies of viral research to reach conclusions about epidemiology, forensics, diagnostics, as well as the origin, evolution, and taxonomy of viruses. Phylogenetic analyses of viruses can be used in the early stages of an epidemic to deduce the relationship of a new virus to other viruses whose sequences and characteristics have been previously described. (Yamada, Onimatsu and Etten, 2006).

An early scheme was developed based on F gene and HN gene sequence diversity as well as the HN gene size, which divided NDV strains into three lineages (Toyoda *et al.*, 1989). Later, the Aldous group studied partial F gene sequences to divide strains into 6 lineages and 13 sub-lineages (Aldous *et al.*, 2003). The result of all these various classification schemes was confusion and contradiction, and there was an obvious need for a unified system of classification. Diel suggested that a genotype based system be adopted, and that the scheme should rely on the complete F gene sequence and that will help researchers better understand NDV epidemiology, evolution, disease control, and diagnostics (Diel *et al.*, 2012). Diel Results revealed Class I viruses have a single genotype divided into three sub-genotypes (1a, 1b, and 1c) and are mainly low-virulent strains and class II viruses have 15 different genetic groupings (I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, and XV).

In order to avoid inconsistencies in naming and classification when dealing with NDV, and in an attempt to deal with the vast amount of data newly made available, international scientists from 29 laboratories, including all OIE reference laboratories for ND, convened in 2014. Their goals included "the establishment of unified criteria for NDV sequence collection and curation" and to "perform comprehensive phylogenetic analyses using the generated datasets" (Dimitrov *et al.*, 2019). The culmination of their efforts was the creation of a unified updated NDV classification system published by Dimitrov *et a,* (2019), which also included reference datasets for other scientists to use for phylogenetic inference, and guidelines such as a cutoff of 10% nucleotide difference for the assignment of genotypes and 5% difference for the assignment of sub-genotypes. Their phylogenetic analysis is based on complete F gene sequences of NDV strains, and includes 1956 sequences. The analysis was based on the general time reversible substitution model, which is the most parameter rich model that incorporates different rates of substation for each

nucleotide pair. A pilot tree (which contains a smaller number of sequences that still provides the same topology of the larger tree) was also provided to decrease the time needed for tree construction when studying NDV samples.

1.6 Newcastle Disease Virus Genotypes

The classification scheme put forth by Dimitrov *et al.*, (2019) divides NDV strains into two classes: class I and class II. Class I is mainly comprised of strains of low virulence, and this class has considerably less genetic diversity than class II, which is reflected in the fact that class I comprises only one genotype with three sub-genotypes (because of their 96% high genetic relatedness (Bello *et al.*, 2018). Most of the class I viruses are isolated from wild birds. The low genetic diversity of this class may be explained by the fact that wild birds are not vaccinated against NDV, so the immune pressure on class I viruses is very low. Additionally, since they are generally avirulent and affect wild birds, there has been relatively little effort put forth to isolate and characterize class I viruses (Dimitrov *et al.*, 2019).

Class II has been found to be much more diverse than class I, containing at least 20 distinct genotypes (I to XXI, genotype XV that contains only recombinant sequences was excluded from the final analyses) distinct genotypes (designated by Roman numerals), and both virulent and avirulent viruses. Under the new classification scheme, some groups that were previously considered sub-genotypes were elevated to the status of new genotypes. Several, but not all, of the genotypes identified in the new classification put forth by Dimitrov contain sub-genotypes, which are designated by Arabic numerals using a numerical-decimal system (eg. VII.1 and VII.2 represent sub-genotypes in genotype VII).

Genotype VI, which includes strains that emerged four decades ago in the Middle East in pigeons (Aldous *et al.*, 2004), is the most diverse NDV genotype. Viruses of this genotype have incredible geographical range as well, and they have been isolated on all continents with the exception of Antarctica.

Genotype VII includes the viruses that caused the fourth and ongoing NDV pandemic, which are included in the sub-genotype VII.1.1. Also found in this genotype is sub-genotype VII.2, which includes viruses that have caused a series of recent outbreaks in

Asia, Europe, and the Middle East, including Israel (Wiseman, Berman and Klement, 2018) and Egypt (Radwan *et al.*, 2013). These outbreaks have been described as a potential fifth pandemic. Since early 2011, Egypt has had NDV epidemics in both vaccinated and unvaccinated flocks, and genotype VII.1.1 was the most prevalent genotype, and was isolated from chickens, pigeons, turquoise, quail and cattle egrets. In addition, genotypes II and XXI.1.1 were also detected in chickens and pigeons. (Ali *et al.*, 2022).

1.7 NDV Vaccines

Control of Newcastle disease depends on strict biosecurity as well as vaccination. The first commercial vaccines against ND were inactivated vaccines, which were made available in 1945 in the United States. These inactivated vaccines were not widely used, being that they were considered expensive and did not completely prevent clinical disease (Dimitrov *et al.*, 2017). Inactivated vaccines for ND in general also have the drawbacks of requiring individual subcutaneous or intramuscular injection, and the shedding of large amounts of virulent challenge, and requiring a withdrawal period for vaccinated birds before human consumption.

In 1948, live vaccines were licensed, but these vaccines were virulent to young birds and thus impractical. Much effort was put forth to find a NDV strain that could be used as a live vaccine without causing unacceptable levels of disease symptoms. Soon, such strains were discovered, including the B1, LaSota, VH and VG/GA strains (avirulent vaccine virus), which had been isolated in the US, and were licensed for use in live vaccines (Dimitrov *et al.*, 2017). These vaccine strains belong to genotype II and share >98% nucleotide identity. A downside to these live vaccines is that they cause some level of clinical symptoms, including a drop in egg production, and are easily inactivated when not refrigerated properly. In addition, these vaccines, while preventing mortality upon challenge with the virus, also do not stop viral replication and shedding, so even vaccinated birds can spread the virus (Samuel *et al.*, 2013). Other groups of vaccines have been developed using strains that are avirulent and have increased thermostability, such as the 1-2 strain. This strain is provided to countries in Asia and Africa by the Australian Centre for International Agricultural Research for the production of vaccines to be used in villages

(Copland and Alders, 2005). These vaccines also do not prevent viral shedding (Susta *et al.*, 2015).

In addition to traditional inactived and live vaccines, several new techniques have also been used to create vaccines against NDV. These techniques include the development of recombinant vaccines using the *Fowlpox virus* (FPV) expressing the NDV F or HN protein. However, these cannot be applied through mass methods, and there is a significant decrease in efficacy of these vaccines in the case of previous exposure to FPV, which is common. Other vectored vaccines used the herpesvirus of turkeys (HVT), and while two such vaccines are used internationally, they are impractical in areas where ND is endemic because they must be kept in liquid nitrogen and require four weeks for immunity to be achieved (Palya *et al.*, 2012).

Reverse genetics has been used to create vaccines that are identical to the circulating virulent NDV strain but altered slightly to decrease virulence. These antigenically matched vaccines result in a higher immune response to the challenge virus as well as decreased viral shedding upon infection. Recent recombinant vaccines using a LaSota backbone have been shown to also require lower doses to result in decreased mortality rates compared to traditional vaccines (Cardenas-Garcia *et al.*, 2015). The production of recombinant vaccines that are antigenically identical to circulating NDV strains requires a better understanding and surveillance of circulating strains and their global distribution. Also, a difficulty in the production of these vaccines is the biosecurity level required in labs that work with virulent NDV strains, as most vaccine producers, especially in the developing world, do not have the necessary biosecurity level required for their production.

2. CHAPTER TWO

2. Problem Statement and Objectives

2.1 Problem Statement

Newcastle disease is one of the most economically devastating diseases to affect the poultry industry, and is designated a notifiable disease by the World Organization for Animal Health (OIE). In order to keep the disease under control, government authorities in numerous countries enforce vaccination and surveillance protocols. Nevertheless, NDV outbreaks have occurred in the surrounding countries in recent years. It is reported that particularly virulent isolates belonging to two newly identified sub-genotypes of genotype VII (VIIh and VIIi, cooresponding to VII.2. in Dimitrov's new classification) have been spreading rapidly in Asia and the Middle East, in what has been called a potential fifth NDV pandemic. NDV isolates of the sub-genotype VIIi are the most prevalent isolates to cause outbreaks in Israel since 2012 (Miller *et al.*, 2015a). To date, no research papers assessing the NDV situation in the West Bank have been published. This is despite the presence of several factors that increase the risk for the spread of NDV, such as the prevalence of backyard poultry that do not follow any vaccination procedures, and the presence of live bird markets where several species of birds from several areas are kept in close quarters where cross infection can occur. This study is the first study that attempts to evaluate the NDV situation in the West Bank by first confirming the presence of NDV by molecular testing followed by the phylogenetic analysis of the isolates and comparison to isolates found in surrounding countries.

2.2 Objectives

The main objective of the present study is:

To isolate and molecularly characterize NDV from affected birds in the West Bank, and carry out phylogenic analysis of these isolates using the pilot tree put forth by Dimitrov *et al.*, (2019).

Specific Objectives:

1. To confirm the presence of NDV from samples from suspected NDV cases from birds in the West Bank by molecular identification of the partial F gene.

2. To classify the obtained isolates as lentogenic, mesogenic, or velogenic by analysis of the F protein cleavage site amino acid sequences and compare the deduced amino acid sequences of the obtained isolates to the deduced amino acid sequences of isolates from the surrounding countries.

3. To validate if partial F gene sequences used to conduct phylogenetic analysis would recapitulate reference genotypes previously defined by Dimitrov *et al*, (2019) full-length F sequences pilot tree.

4. To construct the pilot tree using regional NDV sequences, which were added to Dimitrov's pilot tree in order to compare full and partial sequences for the purpose of building identical topological trees.

5. To construct the pilot tree using both complete and partial F gene sequences in order to conduct phylogenetic analysis of region isolates as well as the isolates obtained from the West Bank to determine locally circulating NDV genotypes.

3. CHAPTER THREE

3. Materials and Methods

This study was conducted at the Palestine-Korea Biotechnology Center at Palestine Polytechnic University, Hebron, Palestine.

3.1 Sample Collection

3.1.1 Samples from The Central Veterinary Lab – Aroub

Four RNA samples were kindly donated by The Central Veterinary Lab – Aroub, which had been collected in 2018 (**Table 3.1**).

3.1.2 Samples from Live and Freshly-Deceased Birds

Sample collection in this study was conducted by convenience, which relied on being led to potential NDV cases by word of mouth from veterinarians and local farm owners to birds suffering from nervous and/or respiratory symptoms or death. These symptoms mainly consisted of unusual twisting of the neck or cases where a large number of birds of a flock suddenly died. A total of 30 samples were collected from pigeons, chickens, and turkeys that displayed symptoms indicative of a potential infection with NDV. Tracheal swabs were also collected directly from live and freshly-deceased birds from small local farms in Hebron and Bethlehem that were suspected of being infected with NDV. Nine of these produced a positive result for NDV as listed in **Table 3.1**. The birds showed symptoms of Newcastle disease, including loss of appetite, coughing, gasping, nasal discharge, watery bright green diarrhea, and nervous symptoms such as twisting of the neck (**Figure 3.1**).

Figure 3.1: Samples collected from local farms. (A) Sample collection using sterile swabs used to collect samples from trachea. (B) Chickens showing signs of NDV. Both died within two days.

Table 3.1: RNA and Swab Palestinian Samples from Diseased poultry

Source	Sample Name	Host	Date of Collection	Region	Vaccination status
Vaccine	Biovac VIR 105-VH strain		November-2020	Hebron	
Aroub RNA samples	PAL-66	Chicken	Januery-2018	Jerusalem	Unknown
	PAL-458	Chicken	March-2018	Nablus	Unknown
	PAL-1025	Chicken	July-2018	Tubas	Unknown
	PAL-1244	Chicken	September-2018	Jerusalem	Unknown
Tracheal Swab	PAL-7H	Chicken	April-2021	Hebron	vaccinated
	PAL-8T	Turkey	April-2021	Hebron	vaccinated
	PAL-Z3	Chicken	Februery-2022	Hebron	vaccinated
	PAL-B2	Chicken	Februery-2021	Bethlehem	Not vaccinated
	PAL-9H	Chicken	April-2021	Hebron	Not vaccinated

3.2 Identification of Strains

3.2.1 RNA Extraction

Total RNA was extracted from 200µl swab specimens, using QIAamp ® MinElute® Virus Spin kit (Catalogue number.57704) according to the manufacturer's instructions. The swabs were placed into 1.5 ml Eppendorf tubes containing 400µl PBS, and incubated for 15 minutes at room temperature in order to release material from swab into the medium. Afterwards, the swabs were shaken vigorously and squeezed. For RNA extraction, 200µl

of each swab extract was mixed with 25µl QIAGEN protease and 200µl of buffer AL (containing 28µg/ml of carrier RNA), which was pulse-vortexed, and incubated for 15 minutes at 56 °C on a heating block. After that, 250µl of ethanol (96-100%) was added to each sample. After mixing thoroughly by pulse-vortexing and incubation with ethanol for 5 minutes at room temperature, the lysate was transferred onto a QIAamp-column followed by two washes with washing buffer (AW-1 and AW-2). Finally, RNA was eluted in 20µl of AVE elution buffer and stored at -80°C until required.

3.2.2 cDNA Synthesis

cDNA was synthesized in 20µl reaction volumes using SuperScript™ IV Reverse Transcriptase (Catalog number: 18090050) according to the manufacturer's instructions. A 3µl sample of eluted RNA was mixed with 1µl of a 50 ng\ µl stock of random hexamer primer, 1 µl of 10 µM dNTPs and then DEPC- treated water was added up to 13µl. The resulting mixture was incubated at 65°C for 5 minutes to eliminate secondary structures, then chilled on ice. The 5X SSIV buffer was pre-warmed at room temperature before use in the reaction. A 4µl amount of buffer, 1µl DDT, 1µl RNase inhibitor and 1.0 SuperScript reverse transcriptase (200U\µL) were added to the reaction tube. The reverse transcription reaction was performed by incubating reaction tubes at 23°C for 10 minutes followed by 53°C for 10 minutes and terminated by heating at 80°C for 10 minutes. The resulting cDNA was stored at -20°C until required.

3.2.3 Primers

Forward and reverse primer sequences specific for the amplification of the partial F gene (**Figure 3.2**) were obtained from literature (Kant *et al.*, 1997) (**Table 3.2**). Primers were dissolved in 1x TE buffer to a concentration of 100 µM, from which working dilutions were made to a final concentration of 10 µM and these were stored at -20°C until needed.

Table 3.2: Primers used for partial amplification of the NDV F gene. The amplicon length was 362 bp.

Primer	Sequence	Length	Properties
Forward (A)	5`– TTG ATG GCA GGC CTC TTG C – 3`	19 nt	GC:57.9% Tm: 58.4
Reverse(B)	5`-GGA GGA TGT TGG CAG CAT T-3`	19 nt	GC:52.6 % Tm: 55.7

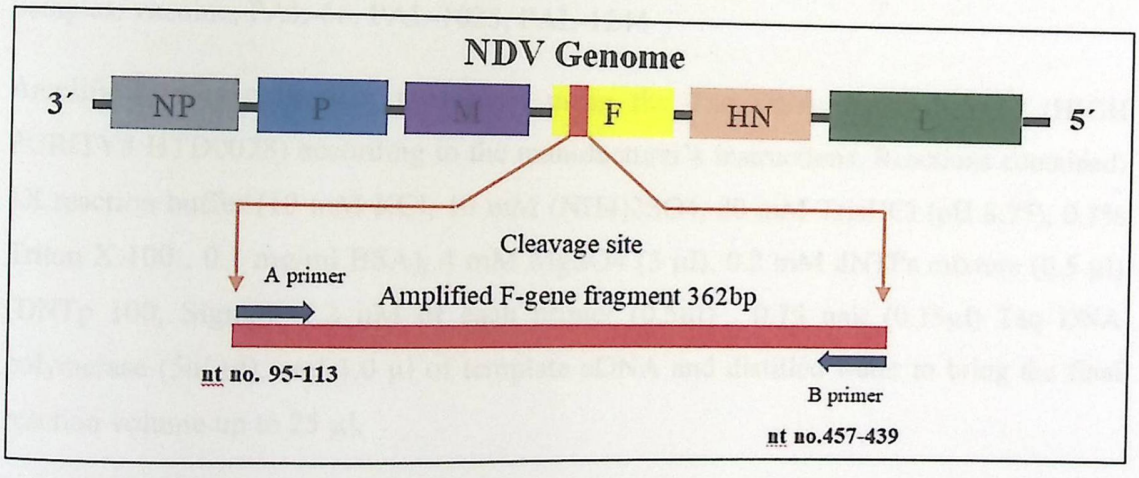


Figure 3.2: PCR target. The red region of the F gene represents the 362 bp region amplified by the primers described above.

3.2.4 Preparation of PCR Controls

NDV vaccine in Palestine market (BIOVAC- VIR 105 live vaccine, VH-strain, Batch: 1-011463, Newcastle disease, SPF) was used as a positive control in PCR reactions. The vaccine powder was dissolved in 1 ml phosphate saline buffer (PBS), and 200µl was taken for RNA extraction, then cDNA synthesis was carried out as described above. The resulting sample was used as positive control in all PCR reactions.

In all PCR experiments the negative control reaction was prepared by mixing all components of PCR without the DNA template, and was named no template control (NTC).

3.2.5 Polymerase Chain Reaction (PCR)

Difficulty was encountered in amplifying the region of interest from the cDNA obtained from some of the samples. Therefore, when standard protocols such as using Taq DNA polymerase kit did not result in the desired results, more sensitive polymerases with their specific conditions were used for such samples as described below. All PCRs used the same primer pairs as described above and all reactions were performed in 25 µl volumes and reactions were carried out using Bio-Rad T100™ Thermal Cycler.

3.2.5.1 Standard Taq DNA polymerase Kit

Samples: vaccine, PAL-66, PAL-1025, PAL-1244

Amplification reaction was performed using the Taq DNA polymerase kit (HIGH PURITY# HTD0078) according to the manufacturer's instructions. Reactions contained: 1X reaction buffer (10 mM KCl, 10 mM (NH4)2SO4, 20 mM TrisHCl (pH 8.75), 0.1% Triton X-100 , 0.1 mg/ml BSA), 4 mM MgSO4 (5 µl), 0.2 mM dNTPs mixture (0.5 µl) (DNTp 100, Sigma), 0.2 µM of each primer (0.5µl) , 0.75 unit (0.15µl) Taq DNA polymerase (5u/ µl), and 1.0 µl of template cDNA and distilled water to bring the final reaction volume up to 25 µl.

Thermocycling conditions were: initial denaturation at 94 °C for 5 min; 35 cycles denaturation at 94 °C for 40 sec, annealing at 53 °C for 40 sec, extension at 72 °C for 40 sec, then a final extension at 72 °C for 5 min, and then a hold at 4°C.

3.2.5.2 Q5-DNA polymerase Kit

Samples: PAL-8T, PAL-458

PCR was performed using the Q5® High-Fidelity DNA Polymerase kit (M0491) according to the manufacturer"s instructions. Reactions contained : the 5X Q5 Reaction Buffer which is detergent-free and contains 2mM Mg++ at the final (1X) concentration, 0.2 mM dNTPs mixture, 0.2 µM each primer, 0.12µl Q5 High-Fidelity DNA Polymerase (0.01 U/µl), 1.0 µl of template cDNA and distilled water to bring the final reaction volume.

Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The **NEB Tm** Calculator was used to determine the annealing temperature when using this enzyme.

Thermocycling conditions were: initial denaturation at 94 °C for 5 min; 35 cycles denaturation at 94 °C for 40 sec, annealing at 67 °C for 40 sec, extension at 72 °C for 40 sec, then a final extension at 72 °C for 5 min, and then a hold at 10°C.

3.2.5.3 OneTaq® Quick-Load® 2X Master Mix with Standard Buffer kit

Sample: PAL-B2

Amplification was performed using OneTaq® Quick-Load® 2X Master Mix with Standard Buffer kit (#M0486S) according to the manufacturer"s recommendations. Reactions contained :1X of OneTaq Quick-Load 2X Master Mix with Standard Buffer (contains dNTPs, MgCl2, buffer components and stabilizers as well as two commonly used tracking dyes for DNA gels), 0.2 µM each primer, 2.0 µl of template cDNA and distilled water to bring the final reaction volume up to 25 µl.

Master mix format contains inert tracking dye for easy and direct loading of PCR products onto gels.

Thermocycling conditions were: initial denaturation at 94 °C for 3 min; 35 cycles denaturation at 94 °C for 40 sec, annealing at 53 °C for 30 sec, extension at 72 °C for 40 sec, then a final extension at 72 °C for 5 min, and then a hold at 10°C.

3.2.5.4 Phusion DNA polymerase kit

Samples: PAL-7H, PAL-9H, PAL-Z3

PCR was performed using Phusion® High-Fidelity DNA Polymerase (#M0530S) according to the manufacturer"s recommendations. Reactions contained : 1X Phusion HF Buffer contains MgCl2 (1.5 mM at the final [1X] reaction concentration), 0.2 mM dNTPs mixture, 0.2 µM each primer, 0.25µl Phusion DNA Polymerase (1.0 units/50 µl reaction),

A final concentration of 3% DMSO is recommended, 1.0 µl of template cDNA and distilled water to bring the final reaction volume up to 25 µl.

Thermocycling conditions were: initial denaturation at 98 °C for 3 min; 35 cycles denaturation at 98 °C for 10 sec, annealing at 65 °C for 30 sec, extension at 72 °C for 30 sec, then a final extension at 72 °C for 5 min, and then a hold at 10°C.

3.2.6 Agarose Gel Electrophoresis

PCR products were run on a 1.5% w/v agarose gel prepared by dissolving agarose in 1x TBE buffer (2.3 g agarose: 150 ml TBE buffer), and stained with ethidium bromide dye. A 5X stock TBE buffer was prepared by mixing 54 g Tris base (Amresco/ 0826), 27 g boric acid (Sigma/ 078K0037), 20 ml 0.5 M EDTA (Alfa Aesar/ 10122546), and distilled water up to 1 L. A 1X dilution of the buffer was used for agarose gel preparation and 0.5X for running buffer in gel electrophoresis.

For each sample, 25 µl of PCR product along with 2 µl of 6X loading dye were mixed and loaded onto 1.5% agarose gel along with 100 bp DNA ladder (Cat. No. DM001-R500). The voltage power ranged between 80 and 120 volts, and amplicons were visualized as bands under UV light and photographed using Bio-Rad Molecular Imager® Gel Doc™ XR System.

The target bands of specific size were excised from the gel and purified with the kit (NucleoSpin® Gel and PCR Clean-up) according to the manufacturer's instructions. The sample was mixed with Binding Buffer NTI and in the case of a cut-out gel band (200µl of NTI per 100 mg gel was added), the sample was heated to dissolve the agarose. The NucleoSpin® Gel and PCR Clean-up column was placed in a 2 ml collection tube (up to 700 µl sample) and centrifuged for 30 seconds at 11000 x g, and the flow-through was discarded. Contamination was removed by simple washing with ethanolic Wash Buffer NT3, followed by centrifugation for 30 seconds at 11000 x g, and the flow-through was discarded. The NucleoSpin® Gel and PCR Clean-up column was placed into a 1.5 ml microcentrifuge tube, and the DNA was bound to the silica membrane of a NucleoSpin® Gel and PCR Clean-up Column. Finally, the pure DNA was eluted under low salt conditions with slightly alkaline Elution Buffer NE (5 mM Tris/HCl, pH 8.5) by added

20.0µl NE and then incubated at room temperature for 1 minute, and then centrifuged at 11000 g for 1 minute. The eluate was retained for sequencing.

3.2.7 Sequencing of Amplified Regions

The purified DNA for Sanger sequencing was sequenced using a BigDye® Terminator v1.1 Cycle Sequencing Kit according to the manufacturer's instructions.

A total of 1-2 µl of purified PCR amplicon was added to a reaction mixture containing 1µl BigDye sequencing buffer at 1X final concentration, 2 µl BigDye, 0.5 µl either forward or reverse primers, and distilled water to bring the final reaction volume up to 10 µl. The sequencing reaction was performed using a thermocycler with the following cycle conditions: initial denaturation at 96 °C for one minute followed by twenty-five cycles at 96 °C for ten seconds, 53 °C for five seconds, and 60 °C for four minutes.

The sequencing product was purified using the BigDye® XTerminator™ purification kit as per the manufacturer's instructions. Briefly, 10 µl of sequencing product was mixed with 45 µl SAM™ solutions and 10 µl BigDye® XTerminator™ solution in each well of an 8-well strip. The plate was closed with AB adhesive cover, followed by vortexing for 30 minutes, then centrifugation at 1000 x g for two minutes. Finally, the plate was loaded on to an Applied Biosystems SeqStudio Genetic Analyzer machine using BigDye®XTerminator™ run module.

Nucleotide sequences were then analyzed and assembled using Sequencher software for Sanger, version 4.1.4

3.2.8 NDV Identification

The raw data from Palestinian sequences were assessed using the Basic Local Alignment Search Tool (BLAST) and compared to other sequences in the GenBank NCBI. A BLAST search was performed to determine the identity of Palestinian sequences.

3.2.9 NDV Classification as Velogenic, Mesogenic, or Lentogenic

Nucleotide sequences for local virus samples were translated to amino acid sequences using the Expasy translation tool, which allows for the translation of a nucleotide

(DNA/RNA) sequence to an amino acid sequence. The deduced amino acid sequences were analyzed for the presence or absence of distinctive basic amino acids in the fusion protein cleavage site for all confirmed Newcastle disease viruses.

3.3 Multiple Sequence Alignment and Analysis

Representative sequences recommended by Dimitrov *et al.*,(2019) for complete F-gene sequences were retrieved from GenBank. Accession numbers are found in Appendix **Table S1** .

Selected sequences from regional countries of genotypes II & VII were also obtained from GenBank which have a complete F-gene sequences where a prior genotype association had been made, were used to build the trees. The sequences for the partial F gene of genotype II isolates from surrounding countries were selected based on a cutoff criterion of sequence identity in BLASTn. Accession numbers are found in Appendix **Table S2.1 and Table S2.2.** All sequences were aligned by the Clustal muscle method using MEGA-X version 10.1.8 software. The alignment was performed with the default gap penalties parameters (Gap Opening Penalty was -400.00 and Gap Extension Penalty was zero).

At the end of the study, a multiple sequence alignment was performed to compare the sequences obtained from genotype II for Palestinian isolates and the other one to compare genotype VII.1 and genotype VII.2 sequences obtained from Palestinian isolates.

3.4 Construction of phylogenetic trees

Dimitrov's working group selected representative full-length F-gene sequences for a class II dataset (termed the "pilot dataset") that would allow for rapid genotyping identification of novel isolates.

Representative sequences from all identified sub/genotypes for the Dimitrov classification scheme (n=125) listed in (**Appendix Table S1**) (Dimitrov *et al.*, 2019) were downloaded from GenBank of the National Center for Biotechnology Information (NCBI).

Phylogenetic trees of aligned sequences were built in MEGA X using the maximum likelihood (ML) method based on the general time reversible (GTR) model with 1000 bootstrap replicates with a discrete gamma distribution was being used to model

evolutionary rate differences among sites, as shown in (**Appendix S4**) and **Figures 4.6, 4.7, 4.8**).

Trees were visualized using FigTree version 1.4.2 to make tree clades colorful as shown in (**Figures 4.2, 4.3, 4.4, 4.5**).

3.5 Pairwise amino acid similarity

Pairwise comparisons of deduced amino acid sequences for Palestinian expected genotype II and genotype VII sequences with group II and group VII sequences from Jordan, Egypt, and Israel (**Table S3.1, Table S2.2**) were computed using the SIAS tool available in the following link: http://imed.med.ucm.es/Tools/sias.html.

3.6 Estimating Time Tree

RelTime with Dated Tips RTDT analyses were conducted using MEGA X by providing the estimated ML tree (shown in **Figure 4.7**) for genotype VII isolates with sampling times.

4. CHAPTER FOUR

4. Results

4.1 Identification and Classification of Strains

4.1.1 PCR, Gel Electrophoresis, and Sequencing

The amplification of the partial F gene region of the isolates produced bands at about 362 bp (**Figure 4.1**), and the purified bands were sequenced.

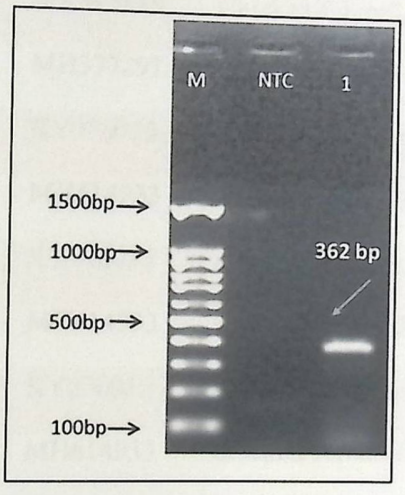


Figure 4.1:Representative image of Agarose gel electrophoresis showing Polymerase chain reaction amplification of the Newcastle disease virus fusion gene using A and B primers, which gave a product size of 362 bp. The amplicons were electrophoresed in 1.5% agarose gel. Lane M:100 bp DNA ladder, Lane NTC: PCR result without the DNA template, Lane 1: PCR result for positive isolates.

4.1.2 Identification by BLASTn

Table 4.1: BLASTn results for isolates most closely related to sequenced Palestinian isolates.

Palestine samples	*Percent Identity*	*Accession Number*	*Isolate name in NCBI*	*Isolate genotype in NCBI*
PAL-Z3	99.67%	MK495888	Chicken/Egypt/Luxor/2012/8	VII.1.1
	99.67%	MH377309	Israel/PHL255913-Turkey/2016	VII.b
PAL-B2	99.68%	MK495888	Chicken/Egypt/Luxor/2012/8	VII.1.1
	99.68%	MH377297	Israel/ PHL257758/2016	VII.b
PAL-8T	98.73%	KY076035	chicken/Pakistan/1002/1A/2015	VII.2
	98.42%	MH614933	Chicken/Jordan/J11-Spleen/2018	VII.2
PAL-1244	98.60%	KY076035	chicken/Pakistan/1002/1A/2015	VII.2
	98.31%	MH614933	Chicken/Jordan/J11-Spleen/2018	VII.2
PAL-1025	97.78%	KY076035	chicken/Pakistan/1002/1A/2015	VII.2
	97.48%	MH614933	Chicken/Jordan/J11-Spleen/2018	VII.2
PAL-7H	99.69%	MN481244	NDV/Chicken/ME5/Egypt/2016	II
	99.69%	MK806396	Chicken/B894/Jiangsu/2018/China	II
PAL-9H	99.45%	MN481244	NDV/Chicken/ME5/Egypt/2016	II
	99.45%	MK806396	Chicken/B894/Jiangsu/2018/China	II
PAL-66	100%	MN481244	NDV/Chicken/ME5/Egypt/2016	II
	100%	MK806396	Chicken/B894/Jiangsu/2018/China	II
PAL-458	99.17%	MN481244	NDV/Chicken/ME5/Egypt/2016	II
	99.17%	MK806396	Chicken/B894/Jiangsu/2018/China	II

BLASTn results clearly show that all the samples collected in Palestine show very high homology to other NDV strains belonging to NDV genotypes II and VII that were attained within the past ten years in **Table 4.1**. The highest degree of homology with isolates in the

GenBank database was found between sample PAL-66 and isolates from Egypt and China, which showed 100% similarity. Similarity of >99% was also found between samples PAL-Z3, PAL-B2, Pal-7H, PAL-9H, and PAL-458 and isolates reported from Egypt, Israel, and China.

4.1.3 Palestinian NDV Classification as Velogenic, Mesogenic, or Lentogenic

Table 4.2: Classification of isolates based on deduced amino acid sequence of the cleavage site (112-117 a.a).

Palestinian Sample	Neucleotide Sequence	Amino acid sequence	Detected genotype	Pathotype
PAL-66	ggg aga cag ggg cgc ctt	GRQGRL	II	Lentogenic
PAL-458	ggg aga cag ggg cgc ctt	GRQGRL	II	Lentogenic
PAL-7H	ggg aga cag ggg cgc ctt	GRQGRL	II	Lentogenic
PAL-9H	ggg aga cag ggg cgc ctt	GRQGRL	II	Lentogenic
Biovac-vaccine	ggg aga cag ggg cgc ctt	GRQGRL	II	Lentogenic
PAL-1025	agg aga cag aaa cgc ttt	RRQKRF	VII	Velogenic
PAL-1244	agg aga cag aaa cgc ttt	RRQKRF	VII	Velogenic
PAL-8T	agg aga cag aaa cgc ttt	RRQKRF	VII	Velogenic
PAL-B2	agg aga caa aaa cgt ttt	RRQKRF	VII	Velogenic
PAL-Z3	agg aga caa aaa cgt ttt	RRQKRF	VII	velogenic

The deduced amino acid sequences of the cleavage site of the F gene revealed that the Palestinian isolates examined in this study consisted of both lentogenic (low virulence) and velogenic (high virulence) strains (**Table 4.2**). The velogenic strains contained a polybasic amino acid pattern that is characteristic of virulent NDV strains, and are the same isolates that BLASTn analysis predicted to belong to genotype VII. The velogenic isolates include two isolates obtained in 2018 (samples PAL-1025 from Tubas and PAL-1244 from

Jerusalem), two isolates obtained in 2021 (samples PAL-8T from Hebron and B2 from Bethlehem) and isolate Z3 obtained in 2022 from Hebron.

4.2 Phylogenetic Analyses

4.2.1 Complete and Partial F Gene Dimitrov's Pilot Trees

In order to determine whether Dimitrov's method for full length F-gene sequence classification could be used to genotype regional and Palestinian partial F-gene sequences, a pilot validation using two trees was done. The first tree was constructed from the same data set that he used. This pilot dataset including representative sequences from all identified sub/genotypes using the complete F gene sequences for the class II dataset was phylogenetically analyzed with the ML method as shown in **Fig 4.2** with the highest log likelihood (-31096.47). The ML approach was used to phylogenetically examine this pilot dataset to demonstrate that the topology derived using entire F gene sequences for the class II dataset would be maintained if partial F gene sequences were utilized. The taxa names include the GenBank identification number, host name, country of isolation, year of isolation, and Diel genotyping.

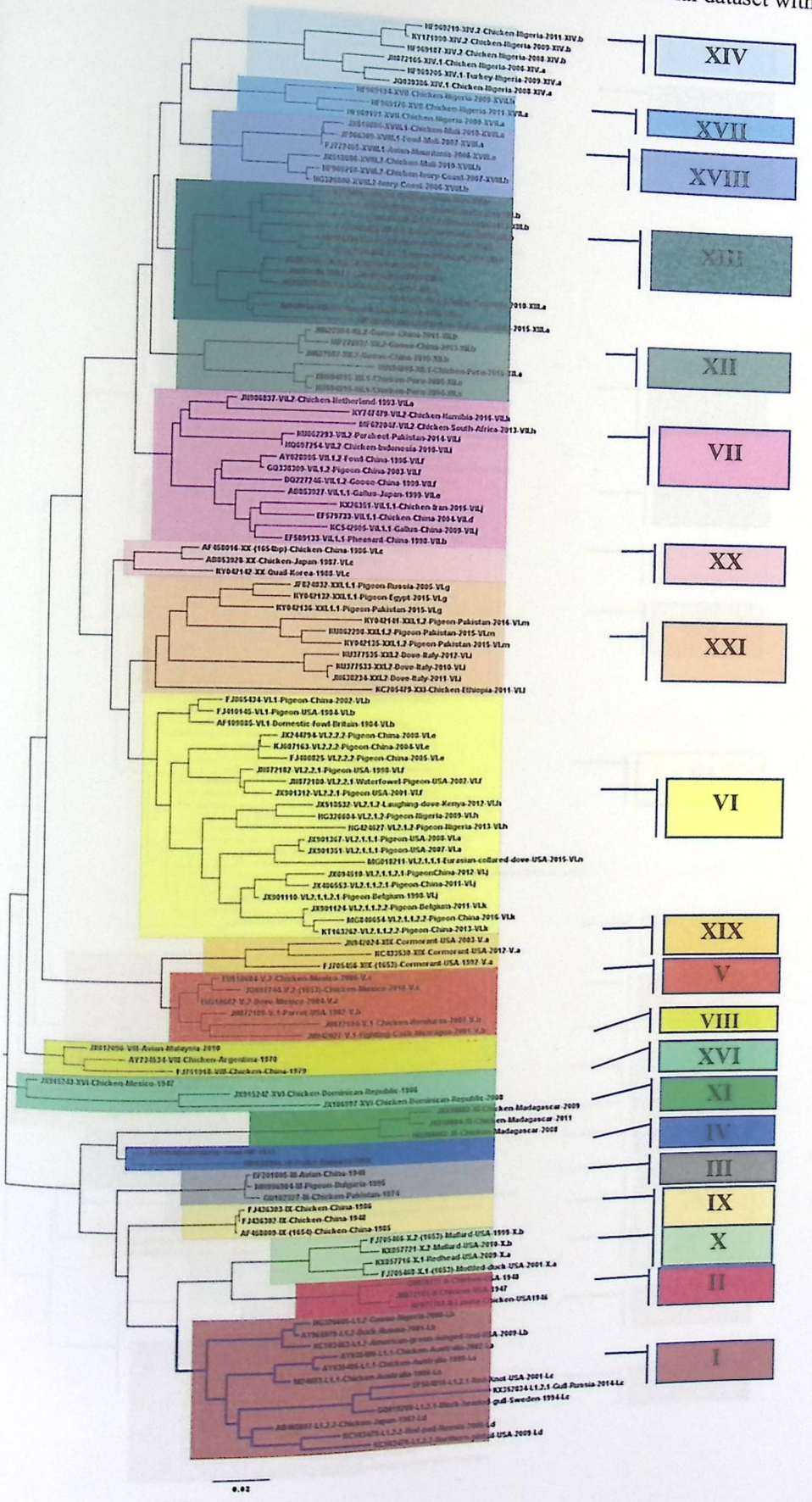
The previously full length-F gene aligned sequences were trimmed to the same size to include only nucleotides from position 95 to 460 (366 bp) to obtain partial F gene sequences congruent with our amplified region, which includes the cleavage site. The tree with the highest log likelihood (-6422.06) is shown in **Fig 4.3.**

The pilot tree for partial F gene sequences successfully reconstituted the same genotype topology as the tree reported by Dimitrov *et al.*, (2019), which used complete NDV F gene sequences. All NDV genotypes were identified and indicated according to color as presented in **Fig 4.2** and **Fig 4.3**. In order to check that trimming reference sequences to match the length of our sequences would not distort the trees, full length and partial sequences from reference isolates were used to build trees and these were compared. Trimming of the sequences to obtain partial F gene sequences obtained a partial F gene pilot tree of identical topology to the complete F gene pilot tree.

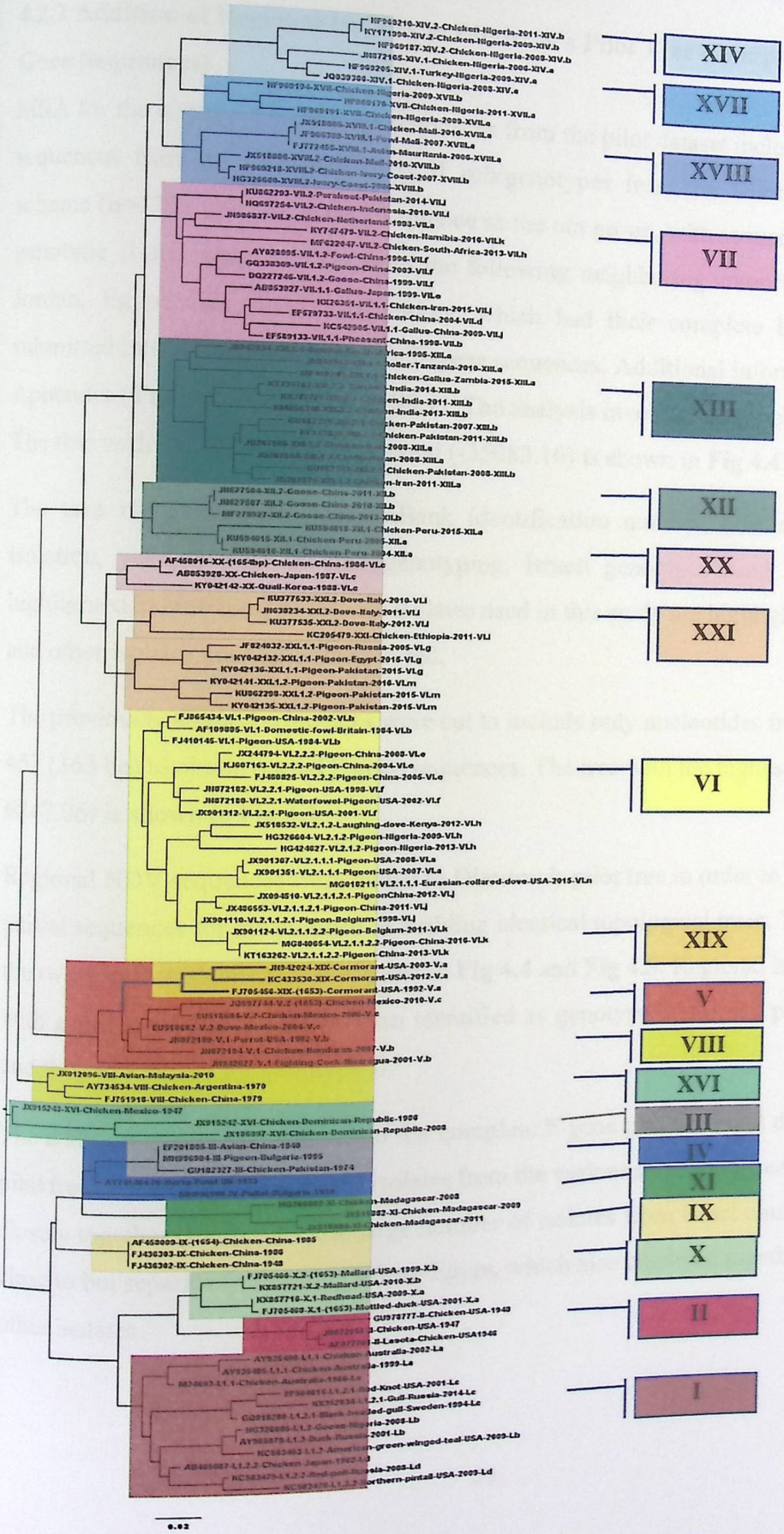
The construction of a phylogenetic tree based on either amino acid or nucleotide sequence necessitates the creation of a data matrix that uses a model for nucleotide or amino acid substitution (also called an evolutionary model). Several models exist of varying complexity depending on the number of parameters taken into account, such as transition and transversion rates, GC content, position of the substitution in the codon, etc. (Caldart *et al.*, 2018). MEGA X is a program that tests 24 different evolutionary models in order to select the one that best fits the input sequence data for maximum likelihood tree construction.

In this study of the partial F gene sequences of the isolates selected by Dimitrov *et al.* for their pilot tree (and cut to the same length as our Palestinian amplification products) the best-fit model with the lowest BIC (Bayesian Information Criterion) scores was found by MEGA X to be the K2+G+I model (i.e., the Kimura 2-parameter model with 4 categories of site-rate heterogeneity under the Gamma model distribution (+G), with invariant sites allowed (+I)). A distinguishing feature of this K2 model is that it takes into consideration different rates of transitions and transversions. The pilot tree to which this model was applied can be found in **APPENDIX S4.2.B**. However, in this study we adopted the GTR + G substitution model, with discrete Gamma distribution, in order to maintain high fidelity to the work of Dimitrov *et al.* (2019). The GTR + G model allows individual probabilities for each possible substitution, and all substitutions are assumed to be reversible (Lam, Hon and Tang, 2010). The two trees constructed using the two different substitution models maintained identical topology almost at the level of genotype and subgenotype, with minor variations observed in bootstrap values as shown in **APPENDIX S4.2.A and B**.

re 4.2.: **Phylogenetic maximum-likelihood tree of NDV complete F gene representing Newcastle disease es of Dimitrov's pilot tree.** There were a total of 1662 positions in the final dataset with 1000 bootstrap replicates.



re 4.3.: **Phylogenetic maximum-likelihood tree of NDV partial F gene representing Newcastle disease isolates of trov's pilot tree.** There was a total of 366 positions in the final dataset with 1000 bootstrap replicates.



4.2.2 Addition of Regional Isolates to Dimitrov's Pilot Tree (Complete and Partial F Gene Sequences)

MSA for the complete F gene for sequences from the pilot dataset included representative sequences from all identified class II sub/genotypes from the Dimitrov classification scheme (**n=125**), and one class I sequence as the out group, with sequences for isolates of genotype II and genotype VII from the following neighboring countries (**n=56**): Israel, Jordan, Egypt, Iraq, Iran, and Turkey, which had their complete F gene sequences submitted into NCBI with complete F-gene sequences. Additional information is found in Appendix (**Table S2.2 and Table S3.1**). The analysis involved 182 nucleotide sequences. The tree with the highest log likelihood (-35083.16) is shown in **Fig 4.4.**

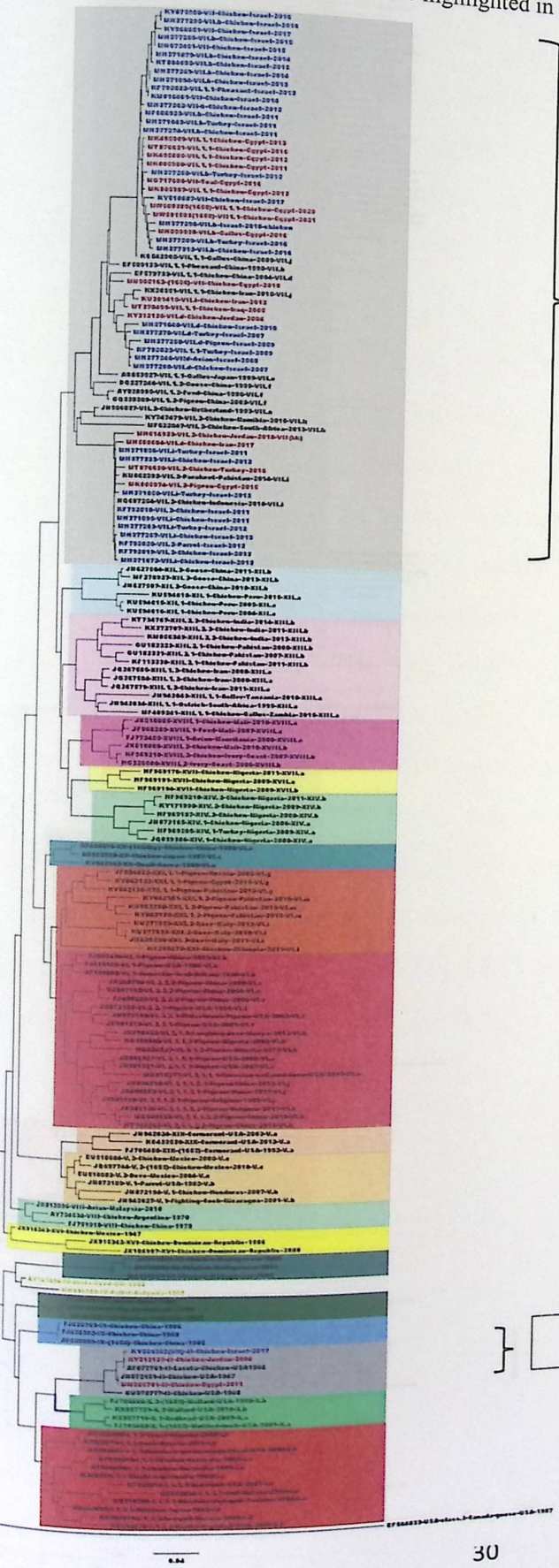
The taxa names include the GenBank identification number, host name, country of isolation, year of isolation and genotyping. Israeli genotypes used in this study are highlighted in blue font, Reference isolates used in this study are highlighted in black font, and other isolates are highlighted in red.

The previously aligned sequences were cut to include only nucleotides from position 95 to 457 (363 bp) to obtain partial F gene sequences. The tree with the highest log likelihood (-6847.06) is shown in **Fig 4.5**.

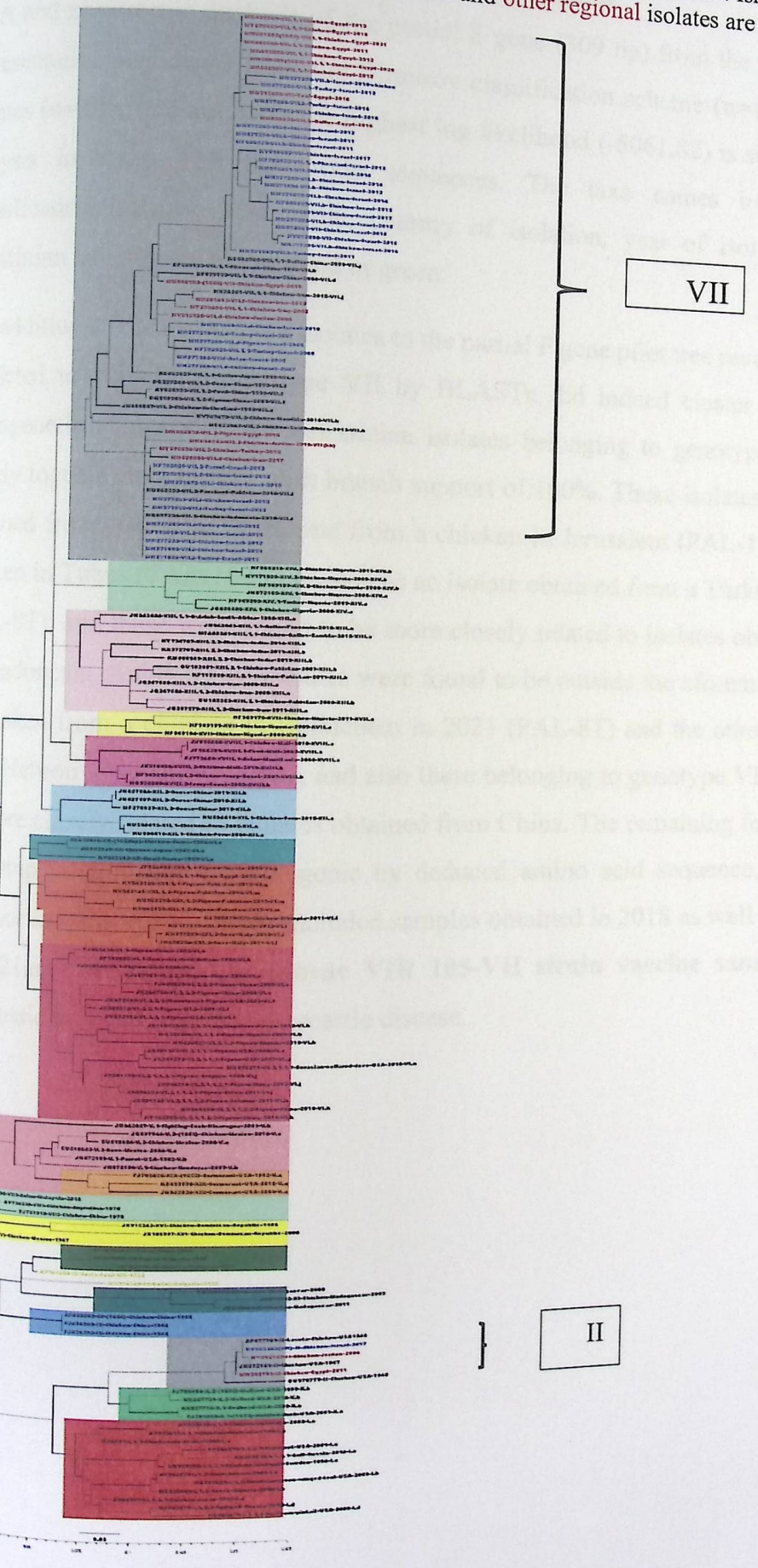
Regional NDV sequences were added to Dimitrov's pilot tree in order to compare full and partial sequences for the purpose of building identical topological trees. The topologies of the two trees are identical as presented in **Fig 4.4** and **Fig 4.5**. Regional isolates associated with genotype VII in NCBI are also identified as genotype VII in the phylogenetic tree, and the same is true for genotype II.

The addition of regional isolates to the complete F gene pilot tree and the partial F gene pilot tree revealed that all regional isolates from the various surrounding countries clustered closely together (**Figure 4.4**). A large number of isolates from Israel clustered together in close to but separate from isolates from Egypt, which also clustered together separate from other isolates.

Phylogenetic maximum-likelihood tree of NDV complete F gene containing Newcastle disease virus isolates of
ot tree in addition to regional isolates. This analysis involved 182 nucleotide sequences with an out-group. There were a
positions in the final dataset with 1000 bootstrap replicates. **Israel** genotypes are highlighted in blue font. **Dimitrov** isolates
in black font and **other regional** isolates are highlighted in red.



Phylogenetic maximum-likelihood tree of NDV partial F gene sequences including Newcastle disease
s of Dimitrov's pilot tree in addition to regional isolates. This analysis involved 181 nucleotide sequences.
total of 366 positions in the final dataset with 1000 bootstrap replicates. Israel isolates are highlighted in
imitrov isolates are highlighted in black font and other regional isolates are in red.

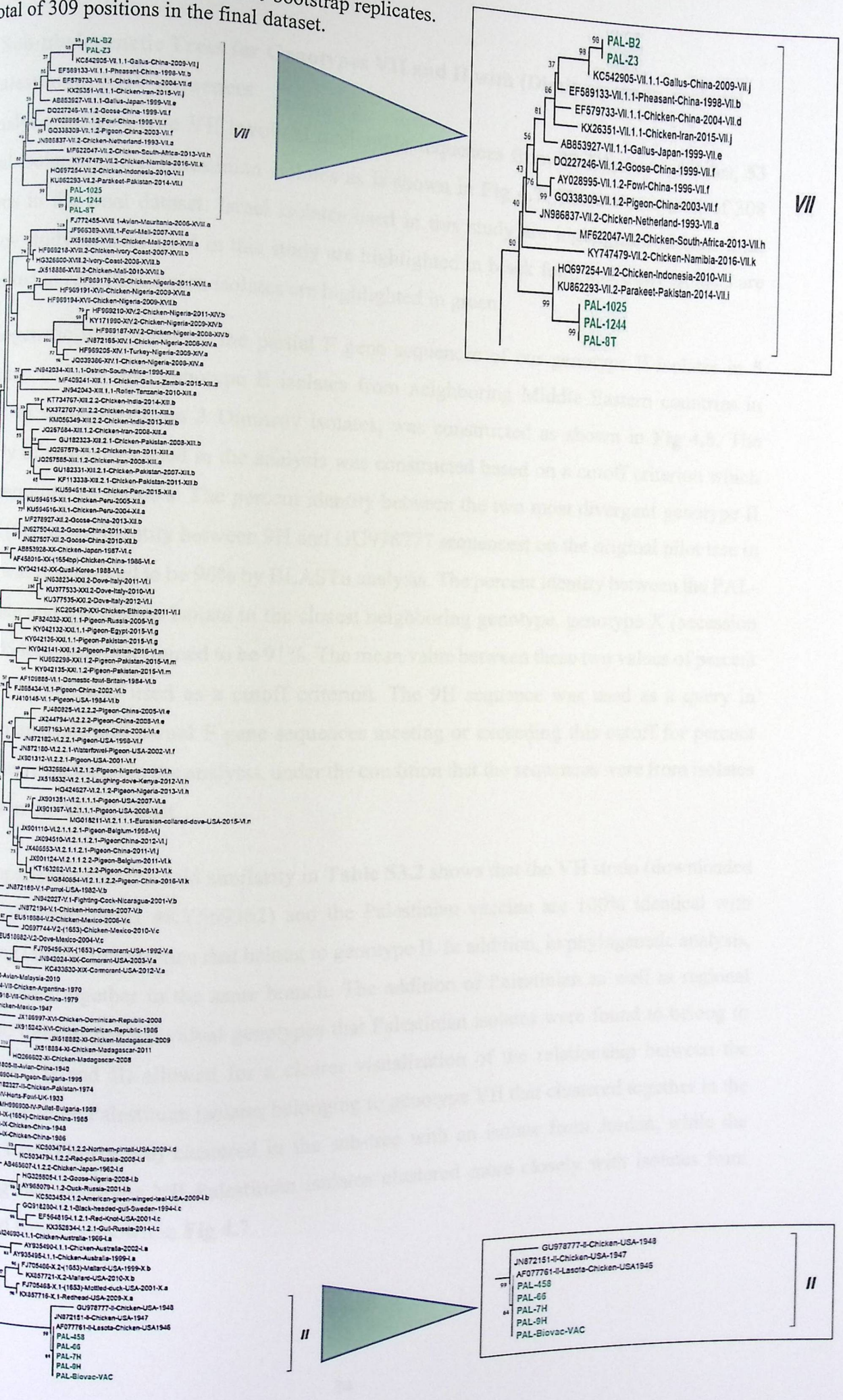


Partial F Gene Pilot Tree with Palestinian Isolates

and phylogenic analysis of the partial F gene (309 bp) from the pilot dataset included sentative sequences from the Dimitrov classification scheme (**n=125**), with Palestinian es (**n=10**). The tree with the highest log likelihood (-5061.82) is shown in **Fig 4.6**. This sis involved **135** nucleotide sequences. The taxa names include the GenBank fication number, host name, country of isolation, year of isolation, and genotype. inian isolates are highlighted in green.

ddition of the Palestinian isolates to the partial F gene pilot tree revealed that the samples ted to belong to genotype VII by BLASTn did indeed cluster in genotype VII by genetic analysis. Three Palestinian isolates belonging to genotype VII clustered very y together in a cluster with branch support of 100%. These isolates include two isolates ed from Aroub in 2018, one from a chicken in Jerusalem (PAL-1244) and one from a n in Tubas (PAL-1025), as well as an isolate obtained from a Turkey in Hebron in 2021 8T), and these were found to be more closely related to isolates obtained from Pakistan donesia. Another two isolates were found to be outside the aforementioned cluster, one ken from a chicken in Bethlehem in 2021 (PAL-8T) and the other was recently taken Iebron (PAL-Z3) in 2022, and also these belonging to genotype VII, and were found to e closely related to isolates obtained from China. The remaining four samples included mples identified as lentogenic by deduced amino acid sequence, and they clustered r in genotype II. These included samples obtained in 2018 as well as samples obtained land the Palestinian **Biovac VIR 105-VH strain vaccine sample** which is a live nic virus vaccine for Newcastle disease.

hylogenetic plot tree with Palestinian isolates
n green font. ML tree with 1000 bootstrap replicates.
otal of 309 positions in the final dataset.



. Sub-phylogenetic Trees for Genotypes VII and II with (Dimitrov study, regional, Palestinian) NDV sequences

analysis for genotype VII involved nucleotide sequences from 13 Dimitrov isolates, 53 nal isolates, and 5 Palestinian isolates as is shown in **Fig 4.7**. There was a total of 308 ions in the final dataset. Israel isolates used in this study are highlighted in blue font. itrov study isolates used in this study are highlighted in black font, and other isolates are lighted in red. Palestinian isolates are highlighted in green.

nylogenetic tree based on the partial F gene sequences of our genotype II isolates in 5 stinian isolates and genotype II isolates from neighboring Middle Eastern countries in tion to sequences from 3 Dimitrov isolates, was constructed as shown in **Fig 4.8**. The mbly of sequences used in the analysis was constructed based on a cutoff criterion which determined as follows: The percent identity between the two most divergent genotype II tes (Sequence Identity between 9H and GU978777 sequences) on the original pilot tree in **4.6** was determined to be 96% by BLASTn analysis. The percent identity between the PAL-solate and the closest isolate in the closest neighboring genotype, genotype X (accession FJ705466), was determined to be 91%. The mean value between these two values of percent tity (93.5%) was used as a cutoff criterion. The 9H sequence was used as a query in ASTn analysis and partial F gene sequences meeting or exceeding this cutoff for percent tity were included in the analysis, under the condition that the sequences were from isolates ined in the Middle East.

comparison of amino acid similarity in **Table S3.2** shows that the VH strain (downloaded n NCBI from Israel #KY569362) and the Palestinian vaccine are 100% identical with ulating viruses in Palestine that belong to genotype II. In addition, in phylogenetic analysis, e clustered together in the same branch. The addition of Palestinian as well as regional ates to trees of the individual genotypes that Palestinian isolates were found to belong to notypes VII and II) allowed for a clearer visualization of the relationship between the ates. The three Palestinian isolates belonging to genotype VII that clustered together in the vious tree **(Figure 4.6)** clustered in the sub-tree with an isolate from Jordan, while the aining two genotype VII Palestinian isolates clustered more closely with isolates from ypt and Israel as shown in **Fig 4.7**.

4.2.4. Sub-phylogenetic Trees for Genotypes VII and II with (Dimitrov study, regional, and Palestinian) NDV sequences

The analysis for genotype VII involved nucleotide sequences from 13 Dimitrov isolates, 53 regional isolates, and 5 Palestinian isolates as is shown in **Fig 4.7**. There was a total of 308 positions in the final dataset. Israel isolates used in this study are highlighted in blue font. Dimitrov study isolates used in this study are highlighted in black font, and other isolates are highlighted in red. Palestinian isolates are highlighted in green.

A phylogenetic tree based on the partial F gene sequences of our genotype II isolates in 5 Palestinian isolates and genotype II isolates from neighboring Middle Eastern countries in addition to sequences from 3 Dimitrov isolates, was constructed as shown in **Fig 4.8**. The assembly of sequences used in the analysis was constructed based on a cutoff criterion which was determined as follows: The percent identity between the two most divergent genotype II isolates (Sequence Identity between 9H and GU978777 sequences) on the original pilot tree in **Fig 4.6** was determined to be 96% by BLASTn analysis. The percent identity between the PAL-9H isolate and the closest isolate in the closest neighboring genotype, genotype X (accession no. FJ705466), was determined to be 91%. The mean value between these two values of percent identity (93.5%) was used as a cutoff criterion. The 9H sequence was used as a query in BLASTn analysis and partial F gene sequences meeting or exceeding this cutoff for percent identity were included in the analysis, under the condition that the sequences were from isolates obtained in the Middle East.

The comparison of amino acid similarity in **Table S3.2** shows that the VH strain (downloaded from NCBI from Israel #KY569362) and the Palestinian vaccine are 100% identical with circulating viruses in Palestine that belong to genotype II. In addition, in phylogenetic analysis, these clustered together in the same branch. The addition of Palestinian as well as regional isolates to trees of the individual genotypes that Palestinian isolates were found to belong to (genotypes VII and II) allowed for a clearer visualization of the relationship between the isolates. The three Palestinian isolates belonging to genotype VII that clustered together in the previous tree (**Figure 4.6**) clustered in the sub-tree with an isolate from Jordan, while the remaining two genotype VII Palestinian isolates clustered more closely with isolates from Egypt and Israel as shown in **Fig 4.7**.

Figure 4.7: Maximum likelihood phylogenetic tree for NDV genotype VII isolates including Palestinian isolates (in green), Israel isolates are highlighted in blue, Dimitrov isolates are in black and other regional isolates are in red. ML tree 1000 bootstrap replicates with the highest log likelihood (-1368.11). There was a total of 308 positions in the final dataset.

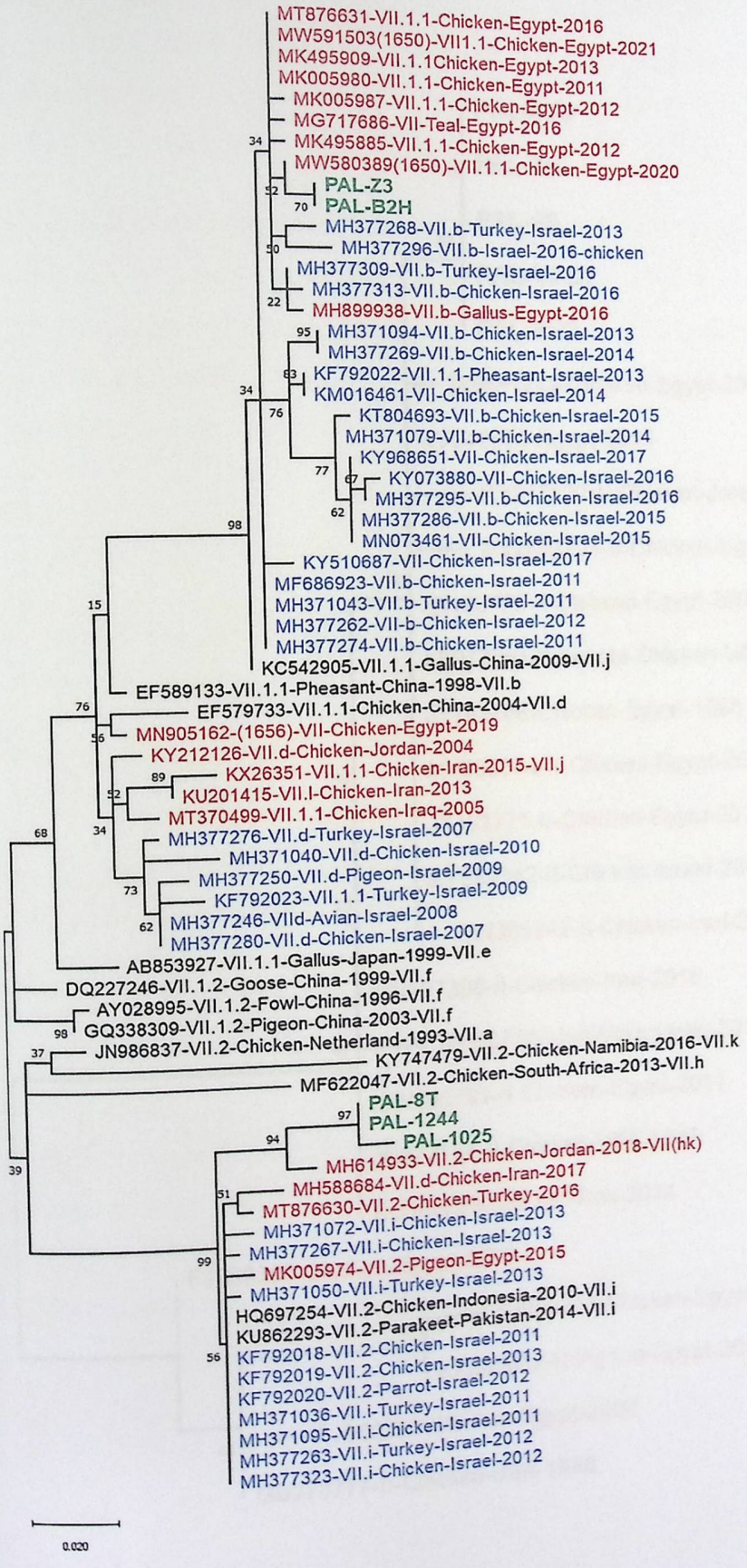
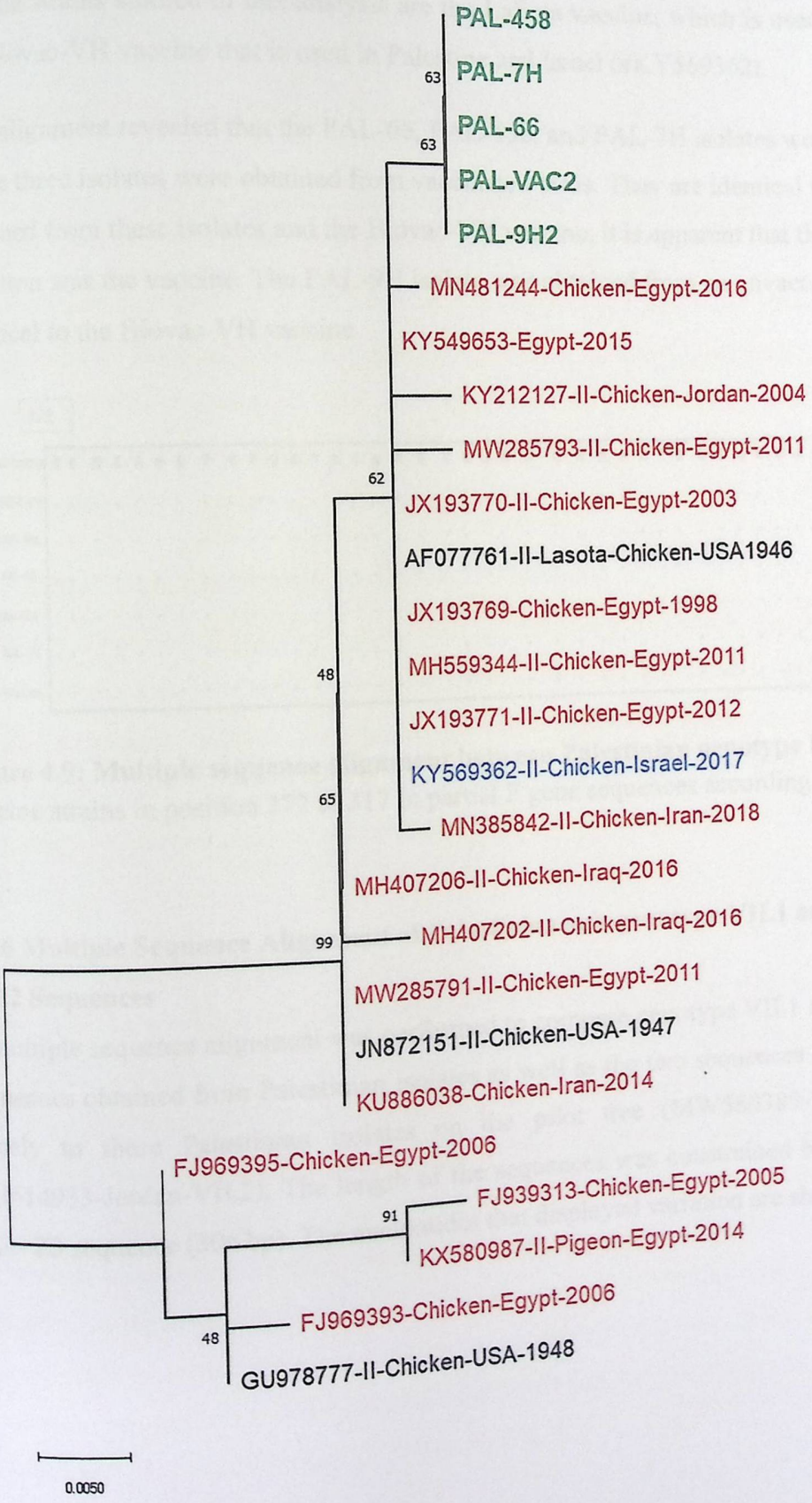


Figure 4.8: Maximum likelihood phylogenetic tree for NDV genotype II isolates including Palestinian isolates (in green). ML tree with 1000 bootstrap replicates. The tree with the highest log likelihood (-549.72). There was a total of 313 positions in the final dataset. This analysis involved 26 nucleotide sequences.



4.2.5 MSA between local genotype II sequences and vaccine sequences

A multiple sequence alignment was performed to compare the sequences obtained from genotype II for Palestinian isolates as well as two vaccine strains as shown in **Fig 4.9**. The vaccine strains studied in this analysis are the LaSota vaccine, which is used worldwide, and the Biovac-VH vaccine that is used in Palestine and Israel (#KY569362).

This alignment revealed that the PAL-66, PAL-458, and PAL-7H isolates were all identical. These three isolates were obtained from vaccinated birds. They are identical to the sequence obtained from these isolates and the Biovac-VH vaccine, it is apparent that the source of their infection was the vaccine. The PAL-9H isolate was obtained from an unvaccinated bird, also identical to the Biovac-VH vaccine.

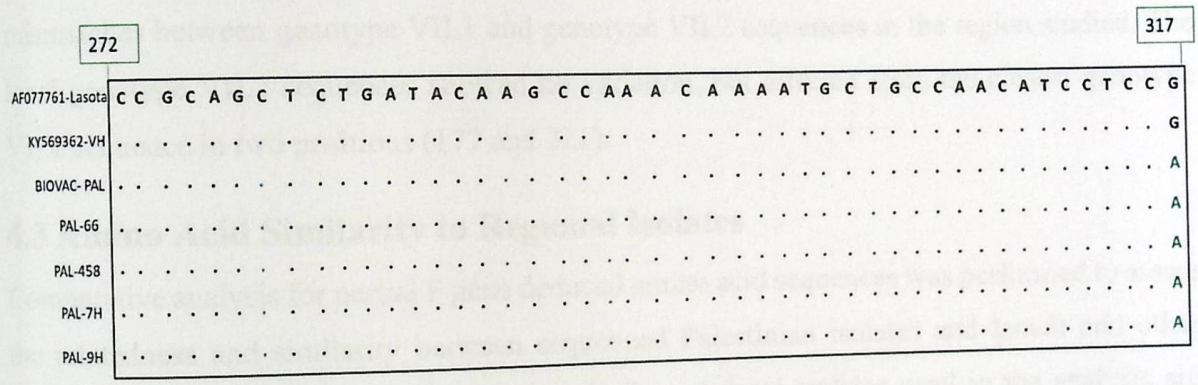


Figure 4.9: Multiple sequence alignment between Palestinian genotype II sequences and vaccine strains in position 272 to 317 in partial F gene sequences according to this study.

4.2.6 Multiple Sequence Alignment of Palestinian sub-genotype VII.1 and sub-genotype VII.2 Sequences

A multiple sequence alignment was performed to compare genotype VII.1 and genotype VII.2 sequences obtained from Palestinian isolates as well as the two sequences that clustered most closely to these Palestinian isolates on the pilot tree (MW580389-Egypt-VII.1.1 and MH614933-Jordan-VII.2). The length of the sequences was constrained by the length of the PAL- Z3 sequence (306 bp). The nucleotides that displayed variation are shown (**Figure 4.10**).

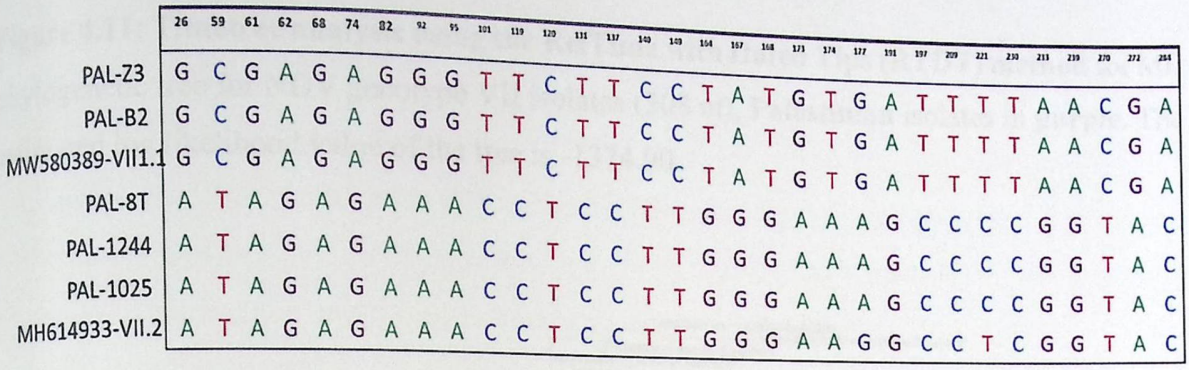


Figure 4.10: Multiple sequence alignment between Palestinian genotype VII sequences with different two sub-genotypes.

The alignment clearly distinguishes between genotype VII.1 and VII.2 sequences. There were no mismatches between the genotype VII.1 isolates in the region studied. There were 32 mismatches between genotype VII.1 and genotype VII.2 sequences in the region studied. The local genotype VII.2 sequences showed no variation, but differed from the closest genotype VII.2 sequence in two positions (177 and 221).

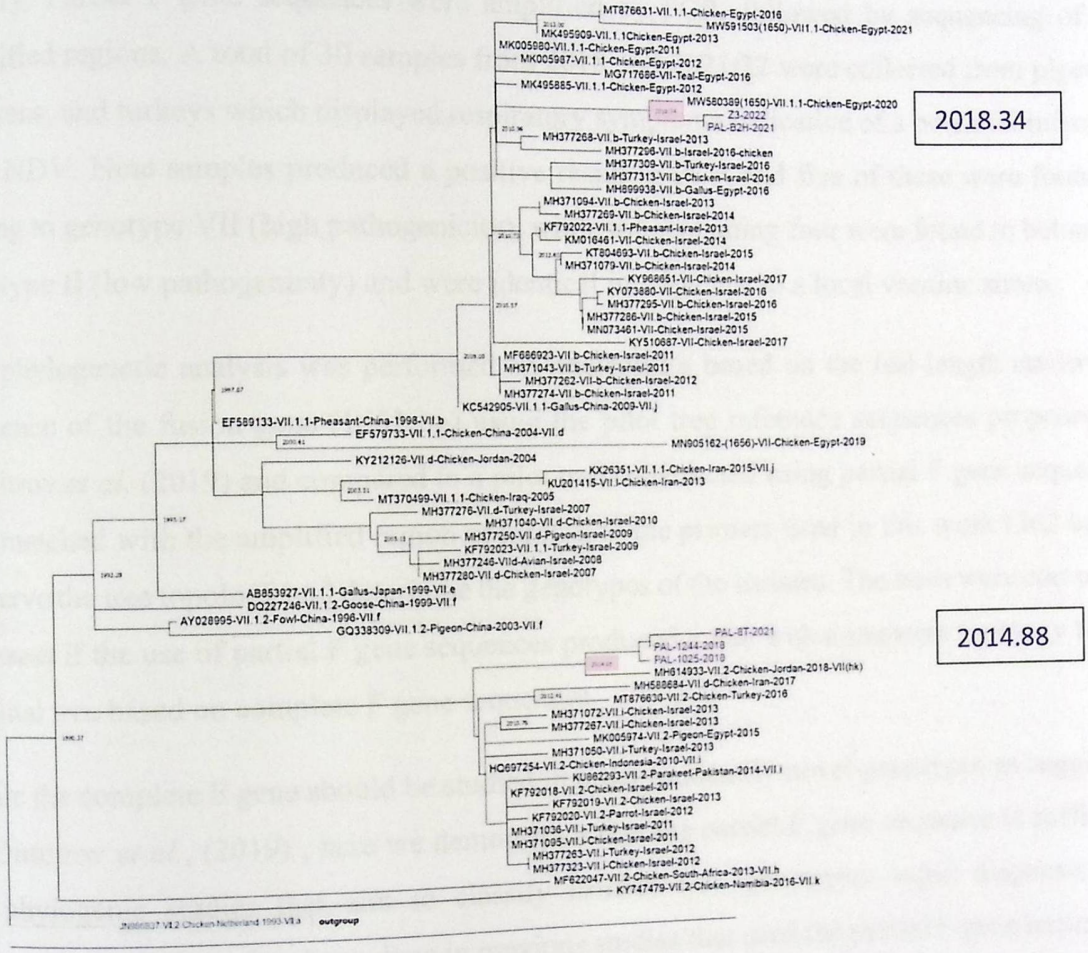
4.3 Amino Acid Similarity to Regional Isolates

Comparative analysis for partial F gene deduced amino acid sequences was performed to assess the relatedness and similarity between sequenced Palestinian isolates and Israeli and other regional sequences. Isolates from Israel and other regional isolates used in the analysis are described in tables in **APPENDIX S3**. Where the deduced amino acid similarity between Palestinian sequences and sequences from regional isolates revealed a very high degree of similarity.

4.4 Time Tree Estimation

A time tree for genotype VII isolates was inferred by applying the RelTime with Dated Tips (RTDT) method to the nucleotide sequence alignment and phylogenetic tree (**Figure 4.7**) whose branch lengths were calculated using the Maximum Likelihood (ML) method and the General Time Reversible substitution model. The branch (JN986837-VII.2-Netherland-1993) was selected to set the root of this tree at this position, with the root sequence being the oldest and the tip sequences being the most recent. The tree is shown in **Figure 4.11**. The time tree was computed using sampling tip dates for 71 taxa that were used as calibration constraints. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.4223)). This analysis involved 71 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

Figure 4.11: Timetree analysis using the RelTime with Dated Tips (RTDT) method for ML phylogenetic tree for NDV genotype VII isolates (308 nt), **Palestinian** isolates in **purple.** The estimated log likelihood value of the tree is -1374.90.



5. CHAPTER FIVE

5. Discussion

This study is the first in Palestine to determine genotypes, and to infer from these pathotypes, of Newcastle disease virus (NDV), which causes a devastating and highly infectious disease in poultry. Partial F gene sequences were amplified by PCR, followed by sequencing of the amplified regions. A total of 30 samples from 2018 and 2021/22 were collected from pigeons, chickens, and turkeys which displayed respiratory symptoms indicative of a potential infection with NDV. Nine samples produced a positive result in PCR and five of these were found to belong to genotype VII (high pathogenicity), while the remaining four were found to belong to genotype II (low pathogenicity) and were identical in sequence to a local vaccine strain.

The phylogenetic analysis was performed on nucleotides based on the full-length nucleotide sequence of the fusion gene (1662 bp) using the pilot tree reference sequences proposed by Dimitrov *et al.* (2019) and compared to a pilot tree constructed using partial F gene sequences that matched with the amplified region according to the primers used in this work (362 bp) to preserve the tree topology and determine the genotypes of the isolates. The trees were compared to assess if the use of partial F gene sequences produced a tree with congruent topology to the original tree based on complete F gene sequences.

While the complete F gene should be studied in order to identify novel genotypes as suggested by Dimitrov *et al.*, (2019) , here we demonstrate that the partial F gene sequence is sufficient for phylogenic studies that aim to classify isolates into genotypes, rapid diagnosis and pathotypic analysis, as has been done in previous studies that used the partial F gene sequences (Dimitrov *et al.*, 2016; Abolnik *et al.*, 2018; Bello *et al.*, 2018; Ghalyanchilangeroudi *et al.*, 2018; EL-Morshidy *et al.*, 2021). Being that abundant NDV nucleotide sequences are available in NCBI, sequence data from GenBank can be used as a reference to compare nucleotide sequences and perform phylogenetic analysis based on the partial F gene in order to predict genotype identity and trace the origin of the virus causing ND in a given region (Saputri, Poetri and Soejoedono, 2021).This suggestion may be valuable in identifying isolates where time is of the essence and in laboratories, particularly in developing countries where funds might be limited.

Maximum likelihood approaches for developing phylogenetic hypotheses depend upon an evolutionary model, and the trees in this study were constructed with the General Time Reversible (GTR) model applied by Dimitrov *et al.* (2019). The GTR family of nested models

for DNA site replacement, which is more complex than other models and assumes distinct rates of substitution for each pair of nucleotides, in addition to varying nucleotide frequencies of occurrence (Choudhuri, 2014). In addition, the GTR family of nucleotide substitution models is the most widely used and useful. However, a family of time-reversible models can be constructed by restricting one or more of the GTR model parameters, and we found that a Kimura model was a better fit than the general GTR model of Dimitrov et al., (2019). Nevertheless, Kimura models are examples of special cases of the GTR model (Jia, Lo and Ho, 2014) and this work adopted the more general GTR model for wider applicability after comparing the outputs of both models and confirming that the trees shared identical topologies with only minor differences in bootstrap values (**figure S4.2**).

This study first determined the viability of using partial F gene sequences to analyze isolates according to the classification scheme presented by Dimitrov *et al.* (2019). The pilot tree for partial F gene sequences successfully reconstituted the same genotype topology as the tree reported by Dimitrov, which used complete NDV F gene sequences. However, sub-genotype categorization was not always definitive, as sub-genotypes VII.2 and V.1, comprised monophyletic clades using complete F-gene sequences (**Fig 4.2**), as described by Dimitrov *et al.* (2019), which broke down when using the partial sequence of 366 nucleotides (**Fig 4.3**). While there were differences in sub-genotype topology between the pilot trees based on partial and full F gene sequences, all Dimitrov reference isolates were classified as being of the same sub-genotype in both trees, whether based on complete or partial F gene sequences. In the next stage, to confirm the robustness of the genetic grouping obtained by the partial F gene sequences, phylogenetic analysis was conducted based on the complete F gene.

Due to the great predictive value of a short protease cleavage site in the F gene product for determining virulence, many investigations focused on partial sequences of the F gene and used these for epidemiology. The partial sequence of the fusion protein surrounding the location where the F0 precursor is broken into F1 and F2 fragments is sufficient for molecular virulence assessment (Suarez, D., Miller, P., Koch, G., Mundt, E. and Rautenschlein, S., 2020).

Phylogenetically, NDV isolates are divided into two groups: class I (low virulence; usually found in wild birds) and class II (very diverse in poultry and wild birds and having a wider range of virulence) with genotypes designated I-XXI (Dimitrov *et al.*, 2019). Using the pilot

tree proposed by Dimitrov (2019) the Palestinian samples in this study were found to belong to two different genotypes of NDV Class II: genotype II, and genotype VII.

The phylogenetic analysis based on partial F gene sequences of genotype II isolates further validates the viability of the use of the partial F gene sequence for identification and analysis of NDV isolates, which is particularly important in epidemiological studies when the goal is to rapidly assess the presence and spread of NDV.

The LaSota vaccine is used worldwide, and the Biovac-VH vaccine is used in Palestine and Israel; both of which are prepared from genotype II NDV strains of low virulence, with the Biovac-VH vaccine strain having a lentogenic cleavage motif of ^{12}-GRQGRL-^{17}. These two vaccine strains have more than 99% identity in nucleotide sequence for the HN and F genes (Shahar *et al.*, 2018). Samples PAL-66 and PAL-458, which were collected in 2018, belonged to genotype II, as well as samples PAL-7H and PAL-9H, which were collected in 2021. The sequences of these samples were all identical (**Fig. 4.9**) to each other and to the Biovac (VH-strain) vaccine strain, from which it is suggested that these infections could have been caused by vaccination. However, while PAL-7H came from a vaccinated flock, PAL-9H came from an unvaccinated flock while the other 2 samples came from flocks with unrecorded vaccination status (Table **3.1**) indicating the possibility of both direct infection as well as the existence of a natural reservoir of infection in wild-birds. It should be noted that as the vaccine is a live one, recovery of viral RNA from vaccinated birds is not strange in of itself, but the presence of clinical symptoms and mortality in vaccinated birds is cause for concern. In addition to biosecurity lapses or misapplication of vaccination protocols, environmental or nutritional factors, bacterial infections, and immunosuppressive viruses may also increase the pathogenicity of vaccination within flocks (Swayne *et al.*, 2019).

Others have also suggested that genotype II NDV strains isolated from poultry have caused infections that could have been derived from vaccination (Abolnik *et al.*, 2004). According to previous research, low-virulence Newcastle disease viruses isolated from non-vaccinated birds were highly similar to vaccine strains, suggesting that vaccine viruses could escape from poultry and be transmitted by free-flying wild birds, perhaps aiding in their spread into the environment (Cardenas Garcia *et al.*, 2013). Wild birds, chickens, and domestic fowl from North and South America, Africa, Asia, and Europe have all been reported to have isolates of this genotype (Bello *et al.*, 2018).

Live vaccinations are frequently given in large doses, either as a spray or in the form of drinking water which likely leads to environmental contamination (Brown and Bevins, 2017). The dose of live ND vaccines provided has a direct relationship with their effectiveness. The challenges connected with vaccinating free-roaming birds, especially those of varying ages, as well as issues maintaining the cold chain to retain the thermo-labile antigens in the vaccines, are all difficulties in the vaccination protocol. Chickens with insufficient immunization frequently become ill and die. The presence of antibodies (including maternal) in birds, can neutralize the vaccine and reduce efficacy (Dimitrov *et al.*, 2017).

Interestingly, it is noted that all of the our lentogenic field isolates were sourced from commercial vaccines, whereas Israel apparently does not have such a high degree of infection from the vaccine, as only one genotype II sequence has been deposited into NCBI from Israel. The reason for this difference in outcome may be due to the strict vaccination protocols that are enforced in Israel against NDV compared to the West Bank. Israeli protocols dictate that a live vaccine be administered on the day of hatching by spray, followed by individual subcutaneous injection of an inactivated vaccine on days 10-12, and yet another aerosol administration of live vaccine at day 17-21(Wiseman and Berman, 2017). On the other hand, in the West Bank in the farms from which samples were taken, vaccine is administered by mass vaccination through drinking water (Personal communication). This may result in birds receiving unequal doses, where some birds receive a high dose that causes symptoms and other birds receiving a low dose that provides no protection. Farms are also located in close proximity to each other, which increases the possibility of cross contamination between farms occurring.

Five of the Palestinian samples are identified by genotype VII, as shown in figure **4.6**, which is grouped into two separate clades with different subtypes. Genotype VII viruses are the most commonly documented in ND outbreaks in poultry, pets, and wild birds all over the world (Wesula Lwande *et al.*, 2021) until the recent pandemic (Clemmons, Alfson and Dutton, 2021) and have been linked to a number of economically significant disease outbreaks in Asia, the Middle East, and areas of America and South Africa since the year 2000. Two samples, PAL-B2 and PAL-Z3, belong to the sub-genotype VII.1.1. The NDV strains responsible for the fourth pandemic belong to sub-genotype VII.1.1 (Dimitrov *et al.*, 2019). In the 1980s, genotype VII viruses first appeared in Taiwan (VIIa and-c) and Indonesia (VIIb), and in the 1990s, they produced the fourth panzootic of ND, which originated in Southeast Asia and expanded to most African countries as well as in South America (Yu *et al.*, 2001; Miller *et al.*, 2015a). These viruses were merged into one genotype (VII.1.1) based on nucleotide distance, combining the

previously identified sub-genotypes VIIb, VIId, VIIe, VIIj, and VIII. An exception is the former sub-genotype VIIf, which was previously classified as a separate sub-genotype VII.1.2 (Sultan *et al.*, 2022). The new merging of these sub-genotypes are according to the Dimitrov scheme (Dimitrov *et al.*, 2019).

The remaining samples (PAL-1025, PAL-1244, and PAL-8T) belong to sub-genotype VII.2, this is the sub-genotype that contains the viruses which are reported to have caused the fifth NDV pandemic (Mapaco *et al.*, 2016; Abolnik *et al.*, 2018; Ghalyanchilangeroudi *et al.*, 2018; Kammon *et al.*, 2018).In fact, given the recent expansion of their host range and geographic distribution, as well as their increasing virulence among vaccinated birds, several of these sub-genotypes (VIIh and VIIi were combined into a single sub-genotype VII.2.) that are involved in the fifth NDV pandemic are those affecting Indonesia, Asia, the Middle East, Europe, and Africa (Bello *et al.*, 2018; Dimitrov *et al.*, 2019; Rabiei *et al.*, 2021; Sultan *et al.*, 2022).

These genotype VII.2 samples also cluster most closely to isolates that are classified as VIIi under old classification systems. This particular sub-genotype has been reported as rapidly circulating in Indonesia, Pakistan, and Israel (Dimitrov *et al.*, 2016) with high mortality rates even in vaccinated flocks. This subtype (VIIi) was also found to be most closely related to NDV isolated last isolated in Indonesia in 1988 and 1990. This suggests that this particular subtype (VIIi) did not evolve from other strains that were widely circulating in poultry and subject to isolation and characterization, but evolved from NDV strains that were circulating in a wild reservoir (Miller *et al.*, 2015b), mainly that wild reservoir species can spread NDV across national borders (Turan *et al.*, 2019). As shown in **figure 4.7,** Israeli NDV strains clustered together with Indonesian and Pakistani strains, suggesting rapid spread and epizootic characteristics for sub-genotype VII.2. The spread of this sub-genotype in neighboring regions presents a serious risk to poultry, suggesting that NDV is continuously evolving. Particularly due to market factors present in some West Bank cities, which include "Friday markets" where different species of live birds from different areas are kept in cages in close conditions where infections can pass between birds and then be carried to different areas. These markets as well as the prevalence of backyard poultry may contribute to the maintenance and evolution of NDV strains if they are left out of any government mandated anti-NDV measures.

To validate the sub-genotypes of virulent genotype VII Palestinian isolates a multiple sequence alignment was performed to compare genotype VII.1.1 and genotype VII.2 sequences obtained from Palestinian isolates as well as the two sequences that clustered most closely to these Palestinian isolates on the pilot tree (MW580389-Egypt-VII.1.1 and MH614933-Jordan-VII.2).

The alignment clearly distinguishes between genotype VII.1 and VII.2 sequences. There were no mismatches between the genotype VII.1 isolates in the region studied. There were 32 mismatches between genotype VII.1 and genotype VII.2 sequences in the region studied. The local genotype VII.2 sequences showed no variation.

In Israel, as well as other areas, there have been persistent outbreaks of NDV, despite strict vaccination protocols. From here arises the question as to why vaccinated birds and flocks are affected (the vaccination status for our samples is shown in **table 3.1**). Vaccine breakthrough may be occurring in part as the circulating strains identified in this study, belong to genotype VII while the strain used to produce the widely used vaccines belongs to genotype II resulting in poor cross-immunity between the vaccination and field challenge virus strains due to genetic divergence between the wild virus and vaccine strain. A study looking at comparative efficacy of vaccines of different strains against challenge with genotype VII strains found the vaccines derived from genotype II to be deficient, resulting in mortality rates of up to 66%, and vaccines homologous to the challenge strain with genotype VII resulted in a significantly better outcome (Sedeik *et al.*, 2019). Accordingly, the production of vaccines antigenically matched to circulating strains may become a strategy to control NDV in endemic areas.

The pathogenicity of NDV strains is commonly inferred by examining the amino acid sequences of the cleavage site through amino acids 112 to 117, with highly pathogenic strains (called velogenic) being characterized by multiple basic amino acids and phenylalanine at position 117 (F^{117}) as the first amino acid of the F1 protein (de Leeuw *et al.*, 2003). On the other hand, the F cleavage site of avirulent (lentogenic) strains is monobasic with a leucine residue at position 117. The deduced amino acid sequences of the cleavage site of the F gene revealed that the Palestinian isolates examined in this study consisted of both lentogenic and velogenic strains (**Table 4.2**). The West Bank velogenic strains belonged to genotype VII and all had the same polybasic cleavage motif RRQKRF, whereas most of the virulent NDV strains had this motif at the cleavage site (Choi *et al.*, 2010). The lentogenic strains were genotype II and all had the monobasic motif: GRQGRL.

The deduced amino acid similarity between Palestinian isolates belonging to genotype VII and isolates from Israel revealed a very high degree of similarity, with a mean similarity of 0.969 as shown in **table S3.3**. The very high similarity between the genotype VII isolates found in this study and isolates found in Israel, with similarity reaching 0.97 and 1. This suggests that there is a high degree of exchange of NDV between the West Bank and Israeli areas.

In addition to genotype II and VII isolates found in Palestine, recent studies have reported the presence of isolates from genotypes XXI.1.1 and VI circulating in pigeons and wild birds in Egypt and Israel (Wiseman *et al.*, 2018; Joshi *et al.*, 2021; Naguib *et al.*, 2021), but these were not seen here. Viruses of genotype VI are high pathogenicity and likely to be virulent in poultry.

It may be that a larger survey would pick up more genotypes, but another explanation for these other regional genotypes (VI and XXI) not being isolated in this study is the possibility of mismatches between the primers used in this study and template DNA derived from these other genotypes. To examine this possibility, multiple sequence alignment was performed on the primer pair against the target sequence from all NDV genotypes. The primers employed in this study were evaluated for their ability to amplify target sequences from the various isolate genotypes observed in the surrounding region. Multiple sequence alignment was carried out on all the sequences used in this study, in addition to the genotype XXI.1.1 and VI sequences from Egypt and Israel (MW285790 and KY042134 from Egypt, and MH377302 from Israel), and the primer sequences used in this study for amplification of the partial F gene. Upon examination of the MSA, it is clear that, despite some mismatches between the primers and some of the genotypes (**Appendix S5**), the critical first two nucleotides of the 3' region of both primers are complementary to the target sequences for many genotypes, including genotypes VI and XXI. Therefore, we conclude that the primers used would be capable of amplifying the target sequence of all the genotypes expected to be present in the region and that genotypes II and VII have been present in the West Bank since 2018, without excluding the possibility that a larger survey including wild birds as well as poultry might detect more genotypes.

Recommendations and Future Studies

Based on our experience in this first study assessing the NDV situation in the West Bank, we have several recommendations for future studies. Firstly, because of the great pathogenicity of genotype VII, screening should regularly be carried out for this genotype at a national level. Secondly, the complete F gene for representative members of each of the two genotype VII clades should be performed for finer detail epidemiological mapping and improved timetree analysis. Lastly, the problem of infections despite vaccination needs to be addressed by improved vaccination protocols and/or by lobbying for the licensing of more effective vaccines. Being that the currently used vaccines are proving ineffective in some cases, future studies should revolve around the need for NDV vaccines to be antigenically matched to the circulating strains, which requires persistent surveillance and characterization of circulating NDV.

6. APPENDIX

Table S1: Complete fusion gene "pilot" dataset of class II NDV used in Dimitrov's study. The dataset contains 125 sequences -and 1 sequence outgroup *- and was used to build the tree depicted in Figure 4.2.

No.	Accession No.	Host	Country	Collection Date	Genotype	Diel Genotype	Cleavage site
1	KX26351	Chicken	Iran	2015	VII.1.1	VII.j	RRQKRF
2	EF579733	Chicken	China	2004	VII.1.1	VII.d	RRQKRF
3	KC542905	Gallus	China	2009	VII.1.1	VII.j	RRQKRF
4	EF589133	Pheasant	China	1998	VII.1.1	VII.b	RRQKRF
5	AB853927	Gallus	Japan	1999	VII.1.1	VII.e	RRQKRF
6	AY028995	Fowl	China	1996	VII.1.2	VII.f	KRQKRF
7	GQ338309	Pigeon	China	2003	VII.1.2	VII.f	KRQKRF
8	DQ227246	Goose	China	1999	VII.1.2	VII.f	RRQKRF
9	JN986837	Chicken	Netherland	1993	VII.2	VII.a	RRQKRF
10	KY747479	Chicken	Namibia	2016	VII.2	VII.k	RRQKRF
11	MF622047	Chicken	South Africa	2013	VII.2	VII.h	RRRKRF
12	KU862293	Parakeet	Pakistan	2014	VII.2	VII.i	RRQKRF
13	HQ697254	Chicken	Indonesia	2010	VII.2	VII.i	RRQKRF
14	JX518885	Chicken	Mali	2010	XVIII.1	XVIII.a	RRQKRF
15	JF966389	Fowl	Mali	2007	XVIII.1	XVIII.a	RRQKRF
16	FJ772455	Avian	Mauritania	2006	XVIII.1	XVIII.a	RRQKRF
17	HF969218	Chicken	Ivory-Coast	2007	XVIII.2	XVIII.b	RRQKRF
18	HG326600	Not mentioned	Ivory-Coast	2006	XVIII.2	XVIII.b	RRQKRF
19	JX518886	Chicken	Mali	2010	XVIII.2	XVIII.b	RRQKRF
20	HF969176	Chicken	Nigeria	2011	XVII	XVII.a	RRRKRF
21	HF969191	Chicken	Nigeria	2009	XVII	XVII.a	RRQKRF
22	HF969194	Chicken	Nigeria	2009	XVII	XVII.b	RRQKRF
23	HF969210	Chicken	Nigeria	2011	XIV.2	XIV.b	RRQKRF
24	KY171990	Chicken	Nigeria	2009	XIV.2	XIV.b	RRQKRF
25	HF969187	Chicken	Nigeria	2008	XIV.2	XIV.b	RRRKRF
26	HF969205	Turkey	Nigeria	2009	XIV.1	XIV.a	RRQKRF
27	JQ039386	Chicken	Nigeria	2008	XIV.1	XIV.a	RRQKRF
28	JN872165	Chicken	Nigeria	2006	XIV.1	XIV.a	RRQKRF
29	KT734767	Chicken	India	2014	XIII.2.2	XIII.b	RRQKRF
30	KX372707	Chicken	India	2011	XIII.2.2	XIII.b	RRQKRF
31	KM056349	Chicken	India	2013	XIII.2.2	XIII.b	RRQKRF
32	GU182331	Chicken	Pakistan	2007	XIII.2.1	XIII.b	RRQKRF
33	KF113338	Chicken	Pakistan	2011	XIII.2.1	XIII.b	RRRKRF
34	GU182323	Chicken	Pakistan	2008	XIII.2.1	XIII.b	RRRKRF
35	JQ267585	Chicken	Iran	2008	XIII.1.2	XIII.a	RRQKRF
36	JQ267584	Chicken	Iran	2008	XIII.1.2	XIII.a	RRQKRF
37	JQ267579	Chicken	Iran	2011	XIII.1.2	XIII.a	RRRKRF
38	JN942034	Ostrich	South-Africa	1995	XIII.1.1	XIII.a	RRQKRF
39	MF409241	Chicken	Zambia	2015	XIII.1.1	XIII.a	RRQKRF
40	JN942043	Roller	Tanzania	2010	XIII.1.1	XIII.a	RRQKRF
41	JN627504	Goose	China	2011	XII.2	XII.b	RRQKRF
42	MF278927	Goose	China	2013	XII.2	XII.b	RRQKRF
43	JN627507	Goose	China	2010	XII.2	XII.b	RRQKRF
44	KU594615	Chicken	Peru	2005	XII.1	XII.a	RRQKRF
45	KU594616	Chicken	Peru	2004	XII.1	XII.a	RRQKRF
46	KU594618	Chicken	Peru	2015	XII.1	XII.a	RRQKRF

47	JX901367	Pigeon	USA	2008	VI.2.1.1.1	VI.a	RRKKRF
48	JX901351	Pigeon	USA	2007	VI.2.1.1.1	VI.a	RRKKRF
49	MG018211	Eurasian-collared-dove	USA	2015	VI.2.1.1.1	VI.n	RRKKRF
50	JX094510	Pigeon	China	2012	VI.2.1.1.2	VI.j	RRQKRF
51	JX486553	Pigeon	China	2011	VI.2.1.1.2	VI.j	RRQKRF
52	JX901110	Pigeon	Belguim	1998	VI.2.1.1.2	VI.j	RRQKRF
53	MG840654	Pigeon	China	2016	VI.2.1.1.2	VI.k	RRQKRF
54	KT163262	Pigeon	China	2013	VI.2.1.1.2	VI.k	RRQKRF
55	JX901124	Pigeon	Belguim	2011	VI.2.1.1.2	VI.k	RRQKRF
56	JX518532	Laughing-dove	Kenya	2012	VI.2.1.2	VI.h	RRQKRF
57	HG326604	Pigeon	Nigeria	2009	VI.2.1.2	VI.h	RRKKRF
58	HG424627	Pigeon	Nigeria	2013	VI.2.1.2	VI.h	RRRKRF
59	JX244794	Pigeon	China	2008	VI.2.2.2	VI.e	KRQKRF
60	KJ607163	Pigeon	China	2004	VI.2.2.2	VI.e	KRQKRF
61	FJ480825	Pigeon	China	2005	VI.2.2.2	VI.e	KRQKRF
62	JN872180	Pigeon	USA	2002	VI.2.2.1	VI.f	RRQKRF
63	JX901312	Pigeon	USA	2001	VI.2.2.1	VI.f	RRQKRF
64	JN872182	Pigeon	USA	1998	VI.2.2.1	VI.f	RRQKRF
65	FJ865434	Pigeon	China	2002	VI.1	VI.b	GRQKRF
66	FJ410145	Pigeon	USA	1984	VI.1	VI.b	RRQKRF
67	AF109885	Domestic-fowl	Britain	1984	VI.1	VI.b	GRQKRF
68	JF824032	Pigeon	Russia	2005	XXI.1.1	VI.g	KRQKRF
69	KY042132	Pigeon	Egypt	2015	XXI.1.1	VI.g	KRQKRF
70	KY042136	Pigeon	Pakistan	2015	XXI.1.1	VI.g	RRKKRF
71	KY042141	Pigeon	Pakistan	2016	XXI.1.2	VI.m	RRQKRF
72	KU862298	Pigeon	Pakistan	2015	XXI.1.2	VI.m	RRQRRF
73	KY042135	Pigeon	Pakistan	2015	XXI.1.2	VI.m	RRQKRF
74	KU377533	Dove	Italy	2010	XXI.2	VI.i	RRQKRF
75	JN638234	Dove	Italy	2011	XXI.2	VI.i	RRQKRF
76	KU377535	Dove	Italy	2012	XXI.2	VI.i	RRQKRF
77	KC205479	Chicken	Ethiopia	2011	XXI	VI.l	RRHKRF
78	AF458016	Chicken	China	1986	XX	VI.c	RRQKRF
79	AB853928	Chicken	Japan	1987	XX	VI.c	RRQKRF
80	KY042142	Quail	Korea	1988	XX	VI.c	RRRKRF
81	EU518684	Chicken	Mexico	2006	V.2	V.c	RRQKRF
82	JQ697744	Chicken	Mexico	2010	V.2	V.c	RRQKRF
83	EU518682	Dove	Mexico	2004	V.2	V.c	RRQKRF
84	JN872194	Chicken	Honduras	2007	V.1	V.b	RRQKRF
85	JN942027	Fighting-Cock	Nicaragua	2001	V.1	V.b	RRQKRF
86	JN872189	Parrot	USA	1982	V.1	V.b	RRQKRF
87	JN942024	Cormorant	USA	2003	XIX	V.a	RRQKRF
88	KC433530	Cormorant	USA	2012	XIX	V.a	KRQKRF
89	FJ705456	Cormorant	Argentina	1992	XIX	V.a	RRQKRF
90	AY734534	Chicken	China	1970	VIII	VIII	RRQKRF
91	FJ751918	Chicken	Malaysia	1979	VIII	VIII	RRQKRF
92	JX012096	Avian	Dominican-Dominican-	2010	VIII	VIII	RRQKRF
93	JX915242	Chicken	Mexico	1986	XVI	XVI	RRQKRF
94	JX186997	Chicken	Nigeria	2008	XVI	XVI	RRQKRF
95	JX915243	Chicken	Russia	1947	XVI	XVI	RRQKRF
96	HG326605	Goose	USA	2008	I.1.2	I.b	GKQGRL
97	AY965079	Duck	American-green-winged-teal	2001	I.1.2	I.b	GKQGRL
98	KC503453	American-green-winged-teal	USA	2009	I.1.2	I.b	GKQGRL
99	EF564816	Red-Knot		2001	I.1.2.1	I.c	GKQGRL

100	KX352834	Gull	Russia	2014	I.1.2.1	I.c	GKQGRL
101	GQ918280	Black-headed-gull	Sweden	1994	I.1.2.1	I.c	GKQGRL
102	KC503479	Red-poll	Russia	2008	I.1.2.2	I.d	GKQGRL
103	KC503476	Northern-	USA	2009	I.1.2.2	I.d	ERQGRL
104	AB465607	Chicken	Japan	1962	I.1.2.2	I.d	GKQGRL
105	AY935490	Chicken	Australia	2002	I.1.1	I.a	RRQRRF
106	AY935495	Chicken	Australia	1999	I.1.1	I.a	RRQGRL
107	M24693	Chicken	Australia	1966	I.1.1	I.a	GKQGRL
108	JN872151	Chicken	USA	1947	II	II	GRQGRL
109	AF077761-	Chicken	USA	1946	II	II	GRQGRL
110	GU978777	Chicken	USA	1948	II	II	RRQKRF
111	FJ705466	Mallard	USA	1999	X.2	X.b	EKQGRL
112	KX857721	Mallard	USA	2010	X.2	X.b	EKQGRL
113	KX857716	Redhead	USA	2009	X.1	X.a	GKQGRL
114	FJ705468	Mottled-duck	USA	2001	X.1	X.a	GKQGRL
115	FJ436303	Chicken	China	1986	IX	IX	RRQRRF
116	FJ436302	Chicken	China	1948	IX	IX	RRQRRF
117	AF458009	Chicken	China	1985	IX	IX	RRQRRF
118	EF201805	Avian	China	1940	III	III	RRQRRF
119	MH996904	Pigeon	Bulgaria	1995	III	III	RRQRRF
120	GU182327	Chicken	Pakistan	1974	III	III	RRQRRF
121	JX518882	Chicken	Madagascar	2009	XI	XI	RRRRRF
122	JX518884	Chicken	Madagascar	2011	XI	XI	RRRRRF
123	HQ266602	Chicken	Madagascar	2008	XI	XI	RRRRRF
124	AY741404	Fowl	UK	1933	IV	IV	RRQRRF
125	MH996900	Pullet	Bulgaria	1959	IV	IV	RRQRRF
*	EF564833	Canada-goose	USA	1987	CLASS I	CLASS I	ERQERL

Table S2.1: Palestinian (partial F gene) and published NCBI sequences from neighboring countries considered as genotype II used in building phylogenetic tree in fig 4.8.

No.	Country	Length of sequence	Accession no.	Identity percent	Query cover	Genotype in NCBI
1	Egypt	1662	MN481244	99.45%	99%	-
2	Egypt	1662	MW285793	99.17%	99%	II
3	Egypt	1662	MW285791	99.17%	99%	II
4	Egypt	1662	FJ939313	95.59%	99%	-
5	Egypt	1792	FJ969395	96.14%	99%	-
6	Egypt	1792	FJ969393	95.59%	99%	-
7	Egypt	1661	KY549653	99.45%	99%	-
8	Egypt	513	JX193771	99.45%	99%	II
9	Egypt	506	JX193770	99.45%	99%	II
10	Egypt	514	JX193769	99.45%	99%	-
11	Egypt	440	MH559344	99.45%	99%	II
12	Egypt	531	KX580987	95.87%	99%	II
13	Israel	1661	KY569362(VH)	99.45%	99%	II
14	Jordan	1662	KY212127	99.17%	99%	II
15	Iraq	420	MH407202	98.92%	76%	II
16	Iraq	420	MH407206	99.28%	76%	II
17	Iran	1659	KU886038	99.17%	99%	-
18	Iran	1203	MN385842	99.45%	99%	II
19	Palestine	317	Vaccine-Biovac	-	-	II
20	Palestine	317	Palestine-66	-	-	II
21	Palestine	317	Palestine-458	-	-	II
22	Palestine	317	Palestine-7H	-	-	II
23	Palestine	317	Palestine-9H	-	-	II

Table S2.2: **Palestinian (partial F gene)** and published **NCBI** sequences from neighboring countries (complete F gene) with assigned genotype VII.

NO.	Accession No.	Host	Country	Collection Date	Dimitrov-Genotype	Diel-Genotype	Cleavage site
1	1244-Palestine	Chicken	Palestine	2018	VII		RRQKRF
2	1025-Palestine	Chicken	Palestine	2018	VII		RRQKRF
3	8T-Palestine	Turkey	Palestine	2021	VII		RRQKRF
4	B2-Palestine	Chicken	Palestine	2021	VII		RRQKRF
5	Z3-Palestine	Chicken	Palestine	2021	VII		RRQKRF
6	KY212126	Chicken	Jordan	2004		VII.d	RRQKRF
7	MH614933	Chicken	Jordan	2018	VII.2	VII.i	RRQKRF
8	MK005980	Chicken	Egypt	2011	VII.1.1		RRQKRF
9	MK005987	Chicken	Egypt	2012	VII.1.1		RRQKRF
10	MK495885	Chicken	Egypt	2012	VII.1.1		RRQKRF
11	MK495909	Chicken	Egypt	2013	VII.1.1		RRQKRF
12	MK005974	Pigeon	Egypt	2015	VII.2		RRQKRF
13	MG717686	Teal	Egypt	2016		VII	RRQKRF
14	MH899938	Gallus	Egypt	2016		VII.b	RRQKRF
15	MT876631	Chicken	Egypt	2016	VII.1.1		RRQKRF
16	*MN905162-(1656)	Chicken	Egypt	2019		VII	RRQKRF
17	*MW580389-(1650)	Chicken	Egypt	2020	VII.1.1		RRQKRF
18	*MW591503-(1650)	Chicken	Egypt	2021	VII.1.1		RRQKRF
19	MH377280	Chicken	Israel	2007		VII.d	RRQKRF
20	MH377276	Turkey	Israel	2007		VII.d	RRQKRF
21	MH377246	Avian	Israel	2008		VII.d	RRQKRF
22	MH377250	Pigeon	Israel	2009		VII.d	RRQKRF
23	KF792023	Turkey	Israel	2009	VII.1.1		RRQKRF
24	MH371040	Chicken	Israel	2010		VII.d	RRQKRF
25	MH377260	Chicken	Israel	2011		VII.b	RRQKRF
26	KF792018	Chicken	Israel	2011	VII.2		RRQKRF
27	MH377274	Chicken	Israel	2011		VII.b	RRQKRF
28	MH371025	Chicken	Israel	2011		VII.b	RRQKRF
29	MF686923	Chicken	Israel	2011		VII.b	RRQKRF
30	MH371095	Chicken	Israel	2011		VII.i	RRQKRF
31	MH371036	Turkey	Israel	2011		VII.i	RRQKRF
32	MH371043	Turkey	Israel	2012		VII.b	RRQKRF
33	KF792020	Parrot	Israel	2012	VII.2		RRQKRF
34	MH377323	Chicken	Israel	2012		VII.i	RRQKRF
35	MH377262	Chicken	Israel	2012		VII.b	RRQKRF
36	MH377263	Turkey	Israel	2013		VII.i	RRQKRF
37	MH377267	Chicken	Israel	2013		VII.i	RRQKRF
38	KF792022	Pheasant	Israel	2013	VII.1.1		RRQKRF
39	KF792019	Chicken	Israel	2013	VII.2		RRQKRF
40	MH371094	Chicken	Israel	2013		VII.b	RRQKRF
41	MH377268	Turkey	Israel	2013		VII.b	RRQKRF
42	MH371050	Turkey	Israel	2013		VII.i	RRQKRF
43	MH371072	Chicken	Israel	2014		VII.i	RRQKRF
44	MH371079	Chicken	Israel			VII.b	RRQKRF

45	MH377269	Chicken	Israel	2014		VII.b	RRQKRF
46	KM016461	Chicken	Israel	2014		VII	RRQKRF
47	MN073461	Chicken	Israel	2015		VII	RRQKRF
48	MH377286	Chicken	Israel	2015		VII.b	RRQKRF
49	KT804693	Chicken	Israel	2015		VII.b	RRQKRF
50	MH377295	Chicken	Israel	2016		VII.b	RRQKRF
51	MH377296	Chicken	Israel	2016		VII.b	RRQKRF
52	MH377313	Chicken	Israel	2016		VII.b	RRQKRF
53	MH377309	Turkey	Israel	2016		VII.b	RRQKRF
54	KY073880	Chicken	Israel	2016		VII	RRQKRF
55	KY968651	Chicken	Israel	2017		VII	RRQKRF
56	KY510687	Chicken	Israel	2017		VII	RRQKRF
57	MT370499	Chicken	Iraq	2005	VII.1.1		RRQKRF
58	KU201415	Chicken	Iran	2013		VII.1	RRQKRF
59	MT876630	Chicken	Turkey	2016	VII.2		RRQKRF
60	MH588684	Chicken	Iran	2017		VII.d	RRQKRF

*- These three isolates have been included in my study, although the number of nucleotides in F-gene is less than 1662 nt, because they are the most recent isolates that have been added into NCBI in the regional area.

S3: Amino Acid Similarity to Regional Isolates

S3.1 Comparison of Genotype II Samples and Isolates from Israel, Egypt and Jordan

Table S3.1: Palestinian (partial F gene) and published NCBI sequences from neighboring countries (complete F gene) with assigned genotype II.

NO.	Accession No.	Host	Country	Collection Date	Dimitrov-Genotype	Diel-Genoty	Cleavage site
1	Vaccine-		Palestine	2021	II		GRQGRL
2	66-Palestine	Chicken	Palestine	2018	II		GRQGRL
3	458-Palestine	Chicken	Palestine	2018	II		GRQGRL
4	7H-Palestine	Chicken	Palestine	2021	II		GRQGRL
5	9H-Palestine	Chicken	Palestine	2021	II		GRQGRL
6	KY569362(V	Chicken	Israel	2017	II	II	GRQGRL
7	MW285791	Chicken	Egypt	2011	II	II	GRQGRL
8	KY212127	Chicken	Jordan	2004	II	II	GRQGRL

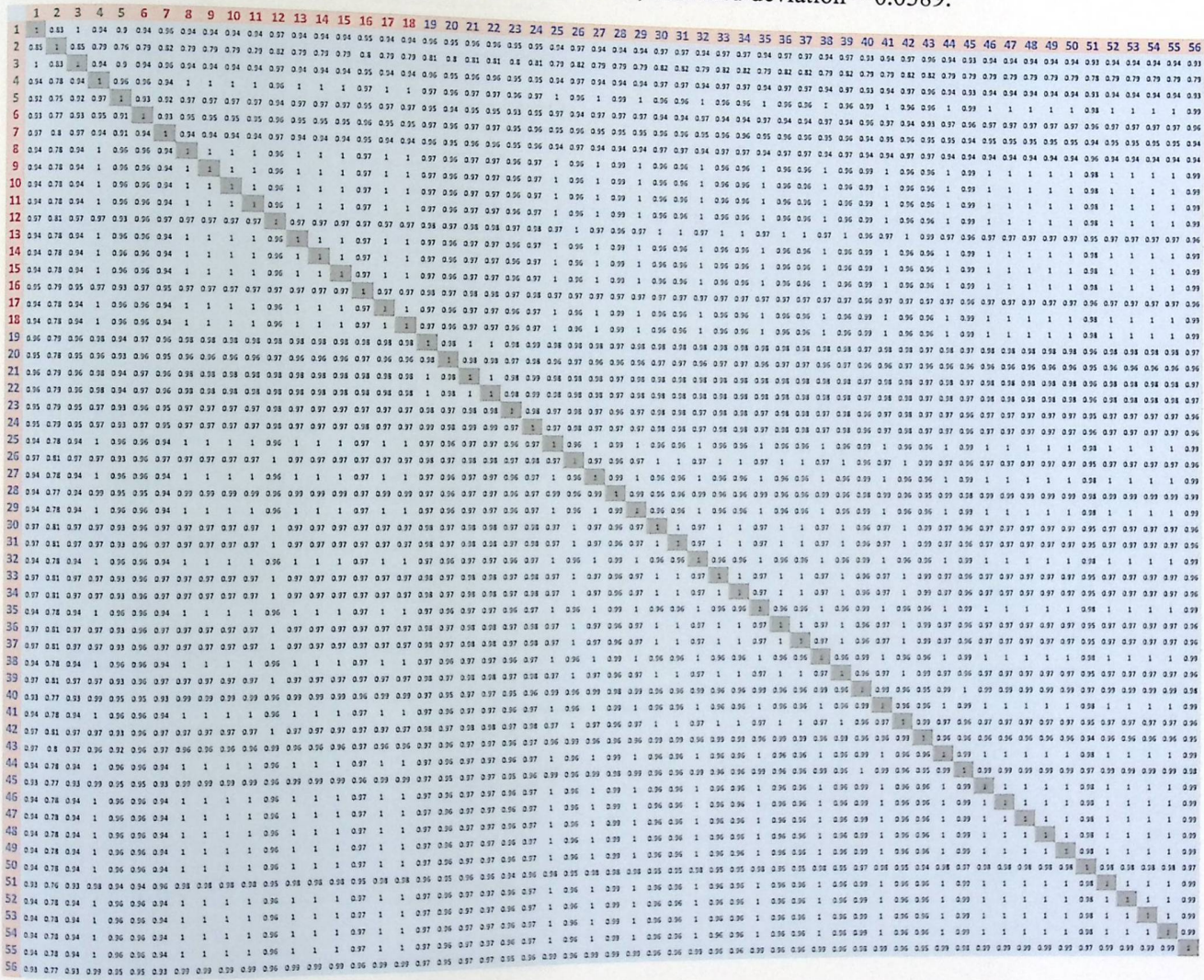
Table S3.2: Global Similarity (Blosum62) between Palestinian genotype II isolates and genotype II isolates from Israel, Egypt, and Jordan (listed in **table S3.1**). Palestinian isolates are in red.

Min = 1, max = 1, mean =1, standard deviation = 0.

	Biovac-vaccine	66-Pal	458-Pal	7H-Pal	9H-Pal	KY569362(VH)-Israel	MW285791-Egypt	KY212127-Jordan
Biovac-vaccine	1	1	1	1	1	1	1	1
66-Pal	1	1	1	1	1	1	1	1
458-Pal	1	1	1	1	1	1	1	1
7H-Pal	1	1	1	1	1	1	1	1
9H-Pal	1	1	1	1	1	1	1	1
KY569362(VH)-Israel	1	1	1	1	1	1	1	1
MW285791-Egypt	1	1	1	1	1	1	1	1
KY212127-Jordan	1	1	1	1	1	1	1	1

S3.2 Comparison of Genotype VII Regional amino acid sequences and Palestinian Isolates

Table S3.3: Global Similarity (Blosum62) between Palestinian genotype VII isolates (1-5 in green) and genotype VII isolates from Jordan and Egypt (6-18 in red) and Israel (19-56 in blue). (All isolates listed in **Table S2.2**). Min = 0.75, Max = 1, Mean = 0.969, Standard deviation = 0.0389.



S4. Phylogenetics constructed by MEGA X with their bootstraps value

S4.1 Complete and Partial F Gene for Dimitrov pilot tree:

Figure S4.1: Complete F Gene (1662 nt) ML Dimitrov Pilot Tree with 1000 bootstrap replicates.

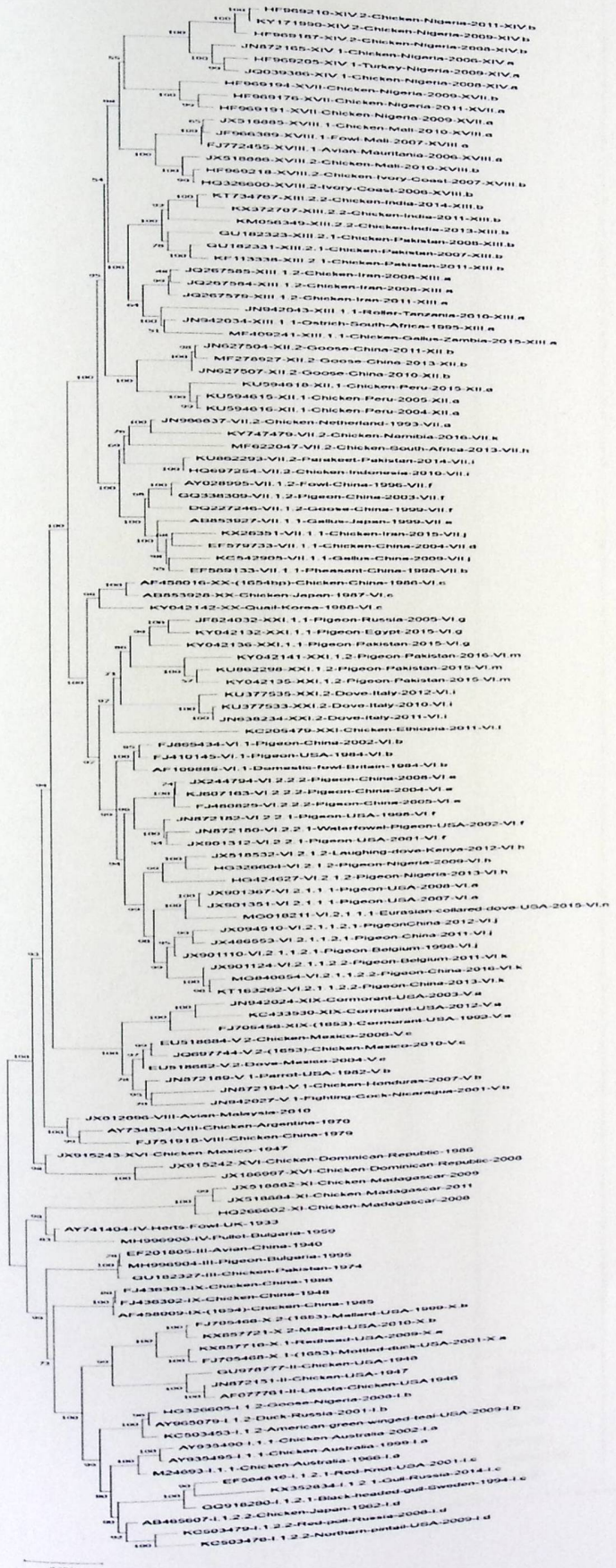
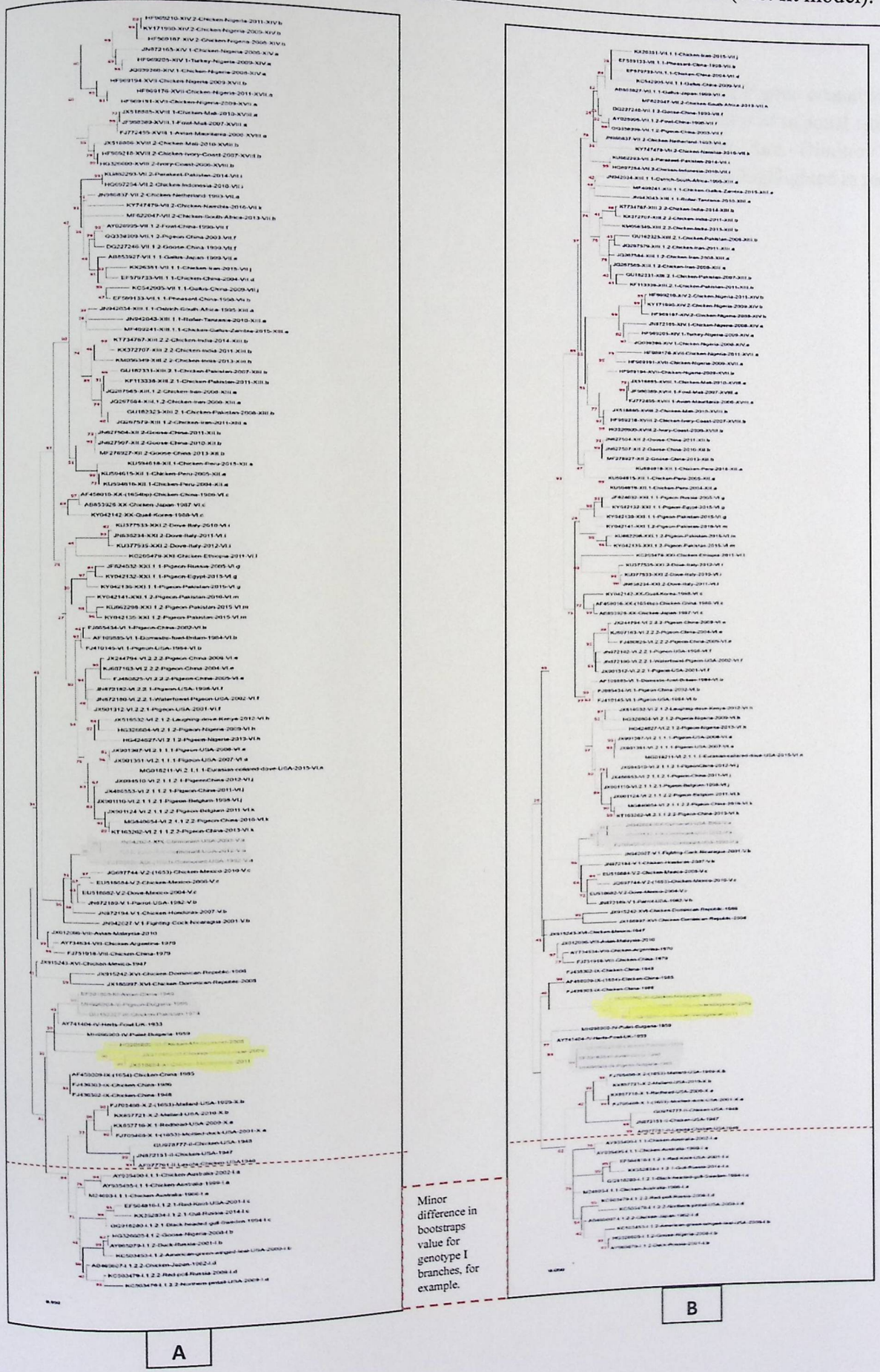


Figure S4.2 Partial F Gene (366 nt) ML Pilot Tree with 1000 bootstrap replicates. A: constructed tree by GTR +G model as Dimitrov work. **B**: constructed tree by K2+G+I model (best fit model).



Construction of Phylogenetic Tree include Dimitrov's Pilot Tree with regional ıtes (complete and partial F gene sequences)

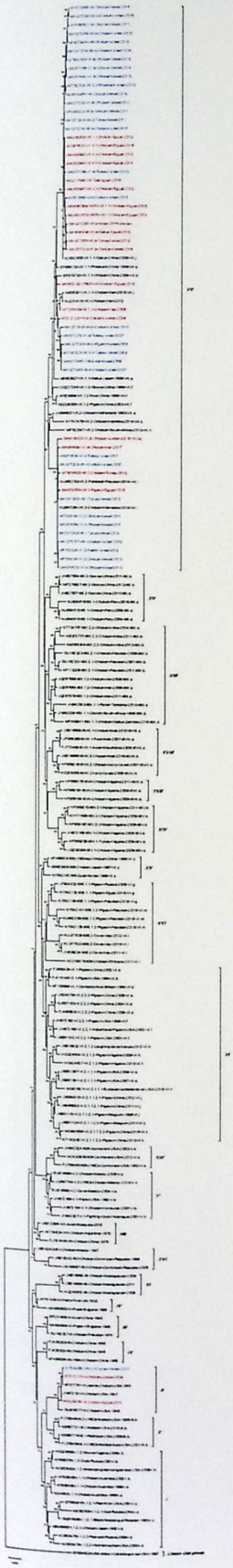


Figure S4.3 Phylogenetic ML tree of NDV complete F gene containing Newcastle disease virus isolates of Dimitrov's pilot tree in addition to regional isolates with an out-group. Israel genotypes are highlighted in blue font. Dimitrov isolates are highlighted in black font and other regional isolates are highlighted in red.

Fig S4.4 Phylogenetic ML tree of NDV partial F gene containing Newcastle disease virus isolates of Dimitrov's pilot tree in addition to regional isolates with 1000 bootstrap replicates. Israel genotypes are highlighted in blue font. Dimitrov isolates are in black font and other regional isolates are in red.

S5. MSA of all NDV full-length F1 sequences with the forward and reverse primers used in this study

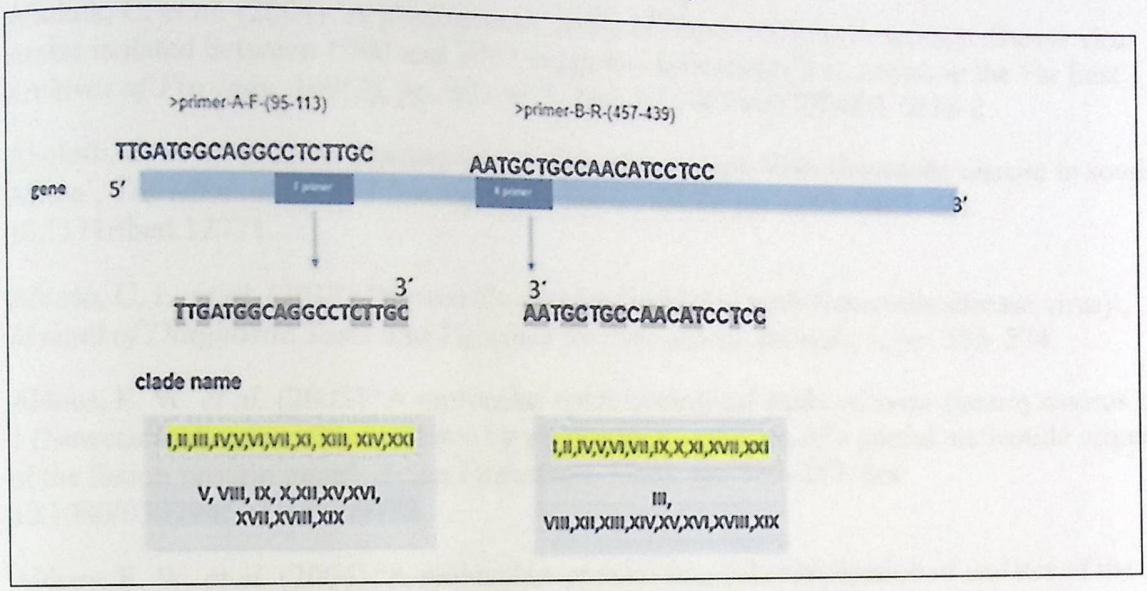


Figure S5.1: The result of MSA of all NDV full-length F sequences with the forward and reverse primers used in this study. These sequences, which include all the Dimitrov (2019) reference sequences, include all genotypes identified to date. The genotypes in yellow indicate genotypes that can be amplified using primers in one or both directions.

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