Original Article

Analysis of a Fragment of the Fusion Gene for Newcastle Disease by DNA Sequencing

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Abstract

Newcastle disease is caused by Avian Paramyxovirus Type 1 (APMV-1), which is found in the genus Avulavirus and family Paramyxoviridae (Suarez et al., 2020). Mild to severe respiratory, visceral, and central nervous system lesions contribute to the illness's devastating impact on vulnerable flocks.

The purpose of this study to analysis of a fragment of the fusion Gene for Newcastle disease virus by DNA sequencing.

Three representative Fusion gene for Newcastle disease virus isolates were sequenced by Macrogen, Korea.

Alignment of the sequences of the three isolated (AN02, AN03 and AN07) against the registered sequences of Newcastle viruses in the database of NCBI were studied, and the result showed that, 94% identity with avian orthoavulavirus 1 isolate By Ck/IR/MAME96/2017, 99% identity with avian orthoavulavirus 1 isolate INDTNC22020 and 86% identity with avian orthoavulavirus 1 isolate INDTNC22020 respectively.

Virulent strain-specific motifs were found in the majority of circulating strains, as shown by partial sequencing of the F gene cleavage site.

Keywords: Fusion Gene, Newcastle Disease, DNA Sequencing.

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INTRODUCTION

Many different types of birds, including chickens, turkeys, and others, are susceptible to Newcastle Disease, a highly contagious and sometimes fatal illness. Depending on how dangerous it is to chickens, it is classified as either extremely pathogenic (velogenic), moderate (mesogenic), or mild (lentogenic) (Tips, 2017). There are two categories of ND virus velogenic strains that account for the virus's global distribution (class I and class II). Class I contains almost all of the low-virulence strains collected from wild ducks across the globe. Class II strains include both low- and high-pathogenic strains isolated from domesticated and wild birds (Birhan *et al*., 2019). The genus Avulavirus is where you'll find NDV; its part of the family Paramyxoviridae and the subfamily Paramyxovirinae. It is a negative-sense RNA virus with a single strand, like its close relatives (Bergfeld, 2017). In spite of the fact that, all NDV strains have a common serotype, they may be divided up into different groups based on their genetic makeup. On the basis of the whole sequence of the F gene, they have traditionally been categorized and described in terms of lineages and sublineages, classes, and genotypes (Tian *et al*., 2020). To clear things up, attempts have been made to identify the best global system of categorization. F gene phylogeny and evolutionary distances form the basis of the approach. Because it is more likely than other internal nucleocapsid genes to exhibit genetic variation, the fusion gene is often employed for comparative study (Susta *et al*., 2015). Rapid and accurate detection of Newcastle disease has been achieved with the use of molecular methods like RT-PCR and PCR (Naf'an *et al*., 2020). Negative and single-stranded RNA viruses may be detected using reverse transcription polymerase chain reaction (RT-PCR). The RT-PCR test may be performed in two distinct ways. The cDNA for a two-tube RT-PCR setup is produced in a separate tube before the PCR experiment is run. On the other hand, cDNA is initially synthesized in a first stage of RT-PCR. In a one-tube PCR process, the reverse transcriptase and polymerase are both inactivated at the same time (El-Tholoth *et al*., 2019). This test is quickly replacing others. When designing an RT-PCR technique, it's crucial to keep in mind a number of critical processes. A first step is the isolation of RNA (Naf'an *et al*., 2020). Using polymerase enzymes, PCR works on the premise that it can simultaneously amplify and distinguish between many target nucleic acids. However, these methods can often only identify a single kind of virus at a time. Viruses that have multiplied in embryos may be detected in both laboratory and clinical specimens using PCR. It is currently the most reliable method for detecting nucleic acids and has the potential to be very sensitive (Worku *et al*., 2022). However, the viruses of interest in this research contain RNA as their nucleic acid, thus PCR cannot be used. Since viral RNA serves as a template for reverse transcription, a particular

oligonucleotide primer and viral RNA must be used to generate single-stranded complementary DNA (cDNA) (Naf'an *et al*., 2020). Due to the rapid turnaround time, molecular methods have become more popular for identifying NDV infection. There are two distinct methods of NDV molecular detection. The first kind seeks to identify any and all NDV genotypes, whereas the second class attempts to distinguish between virulent and non-virulent strains (Moharam *et al*., 2019). Multiple NDV strains have been identified, and they share no genetic similarities. Awad *et al*., (2020) divide NDVs into classes I and II based on their level of genetic variability. NDVs may be detected anywhere because to universal detection primers that target the highly conserved gene M. Using these primers, you won't be able to identify any NDVs at all. As a result, primers specific to gene L were used in tandem with primers specific to gene M for complete NDV genotype detection. Virulence may be identified by PCR experiments with primers specific to the F gene. Pathogenicity is determined by sequencing the PCR product and examining the amino acid sequence of the cleavage site, as defined by vNDV. The considerable diversity of the F gene across NDV strains is a drawback for experiments targeting this gene. As a result, these primers cannot be used to identify certain very dangerous strains (Mphuthi, 2018).

Materials and Methods

Viral RNA extraction

Using Viral Gene-spinTM Viral DNA/RNA Extraction Kit).

Fusion gene sequencing method

F gene confirmation sequencing and phylogenetic tree analyses were carried out using RNA sequencing. We submitted the PCR-positive fusion protein gene to Macrogen Company in Korea to have its RNA sequenced using the AB RNA sequencing technology. Multiple sequence alignment study of the partial fusion protein gene sequence based on Clustal W alignment analysis and the Molecular Evolutionary Genetics Analysis, version 6.0 (Mega 6.0) were used in the DNA sequencing analysis. Maximum Composite Likelihood based upon the UPGMA phylogenetic tree was used to calculate evolutionary distances.

Results

FASTA files of three representative isolates (Sequenced by Macrogen, Korea)

>AN02

CCCAGGGTTAAGGAGGCGTGTTCGAAAGCACAATC
TGAGGCAAATATTGGAACACTGACTGCTTTGCCCA
CTCCTCTTGGTGACTCAAACCGGAAGATCCAAAAG
TCTGTGTCCACATCTGGAGGAAGGAGGCAAAAACG
TTTTATAGGTGCTGTTATTGGCAGTGTAGCTCTTGG
GGTTGCAACAGCGGCACAGATAACAGCAGCTGCGG
GCCTAATACAAGCCAACCAAAATGCCGCCAACATC
CTCCAGCTTAAGGAGAGCATTGCTGCAACCAATGA
AGCTGTGCATGAAGTCACCAACGGATTATCACAAC
TA

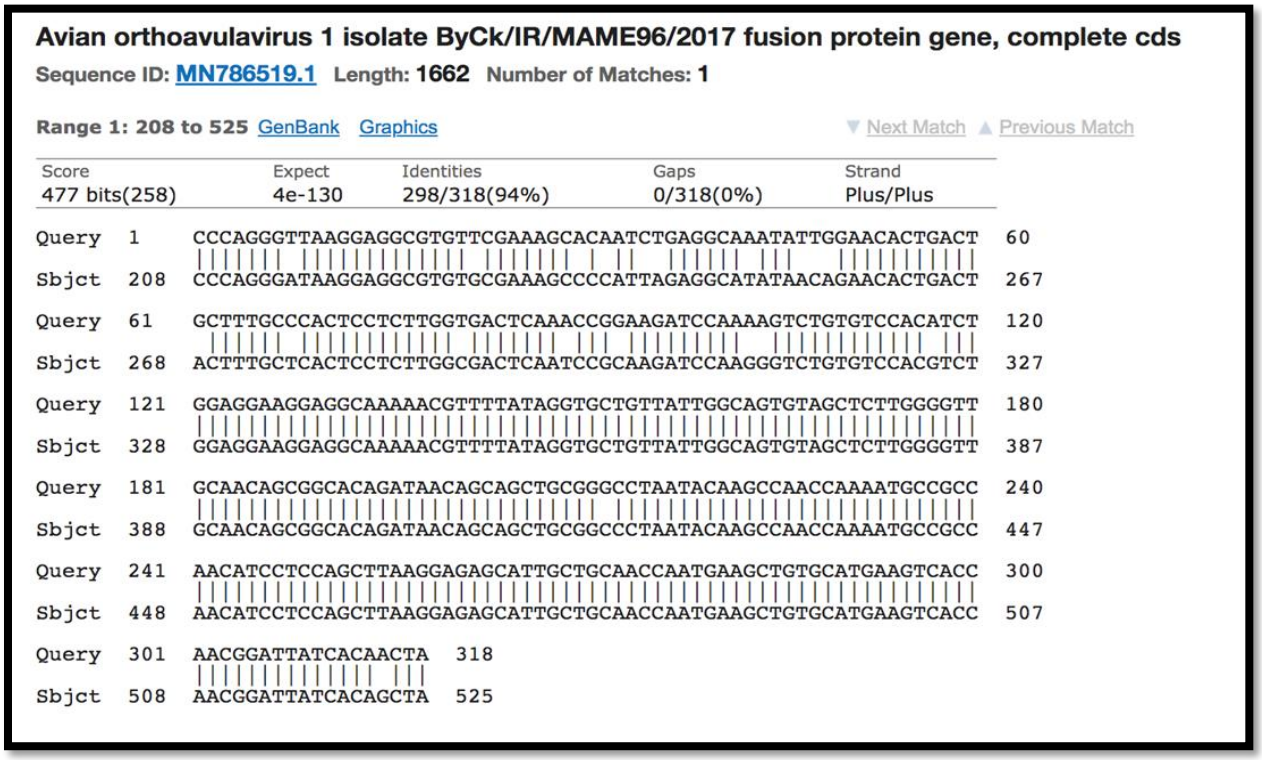
>AN03

ATACACCTCATCCCAGACAGGATCAATCATAGTTA
AGCTCCTCCCGAATCTGCCCAAGGATAAGGAGGCA
TGTGCGAAAGCCCCCTTGGATGCATACAACAGGAC
ATTGACCACTTTGCTCACCCCCCTTGGTGACTCTAT
CCGTAGGATACAAGAGTCTGTGACTACATCTGGAG
GGGGGAGACAGGGGCGCCTTATAGGCGCCTTTATT
GGCGGTGTGGCTCTTGGGGTTGCAACTGCCGCACA
AATAACAGCGGCCGCAGCTCTGATACAAGCCAAAC
AAAATGCTGCCAACATCCTCCGACTTAAAGAGAGC
ATTGCCGCAACCAATGAGGCTGTGCATGAGGTCAC
TGACGGATTATCACAACTAGC

>AN07

GTCCGGGATACTCCATTGTTTGTTTGCCTCTTGCAG
CAGCTGCGGTTGTGGTTACTTGAGAGGAAGCCGTC
AACATATACGCCTCATCGCAGATAGGGTCAATCAT
AAGTAAGCTCCTCCCGAATCTGCCCAAGGATAAGG
AGGCATGTGCGAAAGCACCCTTGGATGCATACAAC
AGGACATTGACCACTTTGCTCACCCCCCTTGGTGAC
TCTATCCGTAGTTTCCAAGAGTCTGTGACTACATCT
GGAGGGGGGAGACAGGGGCGCCTTATAGGTGCCTT
TGTTGGGGATGAGGTGTTTGTGGTTGCAACTGCCTC
TCAAATAACACCGATTCCTGCTCTGACAAAAGCCA
AACAAAATGTTGCCATTATCCTCAGACTTAAAAAT
AGCATTGGCCCACCCAATGAGACTCTGCATCAGAG
CACTGACGTACTAGCACAAC

Alignment of the sequences of the three isolated (AN02, AN03 and AN07) against the registered sequences of Newcastle viruses in the database of NCBI were studied, and the result showed that, 94% identity with avian orthoavulavirus 1 isolate ByCk/IR/MAME96/2017, 99% identity with avian orthoavulavirus 1 isolate INDTNC22020 and 86% identity with avian orthoavulavirus 1 isolate INDTNC22020 respectively. These results were shown in Figures (1), (2), and (3).



Avian orthoavulavirus 1 isolate ByCk/IR/MAME96/2017 fusion protein gene, complete cds

Sequence ID: MN786519.1 **Length:** 1662 **Number of Matches:** 1

Range 1: 208 to 525 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
477 bits(258)	4e-130	298/318(94%)	0/318(0%)	Plus/Plus

```
Query  1    CCCAGGGTTAAGGAGGCGTGTTCGAAAGCACAATCTGAGGCAAATATTGGAACACTGACT  60
            ||||||| ||||||||||||| ||||||| | ||  |||||| |||   ||||||||||
Sbjct  208  CCCAGGGATAAGGAGGCGTGTGCGAAAGCCCCATTAGAGGCATATAACAGAACACTGACT  267

Query  61   GCTTTGCCCACTCCTCTTGGTGACTCAAACCGGAAGATCCAAAAGTCTGTGTCCACATCT  120
             |||||| |||||||||||| ||||||| ||| |||||||||  |||||||||||| |||
Sbjct  268  ACTTTGCTCACTCCTCTTGGCGACTCAATCCGCAAGATCCAAGGGTCTGTGTCCACGTCT  327

Query  121  GGAGGAAGGAGGCAAAAACGTTTTATAGGTGCTGTTATTGGCAGTGTAGCTCTTGGGGTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  GGAGGAAGGAGGCAAAAACGTTTTATAGGTGCTGTTATTGGCAGTGTAGCTCTTGGGGTT  387

Query  181  GCAACAGCGGCACAGATAACAGCAGCTGCGGGCCTAATACAAGCCAACCAAAATGCCGCC  240
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  388  GCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTAATACAAGCCAACCAAAATGCCGCC  447

Query  241  AACATCCTCCAGCTTAAGGAGAGCATTGCTGCAACCAATGAAGCTGTGCATGAAGTCACC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448  AACATCCTCCAGCTTAAGGAGAGCATTGCTGCAACCAATGAAGCTGTGCATGAAGTCACC  507

Query  301  AACGGATTATCACAACTA  318
            ||||||||||||| |||
Sbjct  508  AACGGATTATCACAGCTA  525
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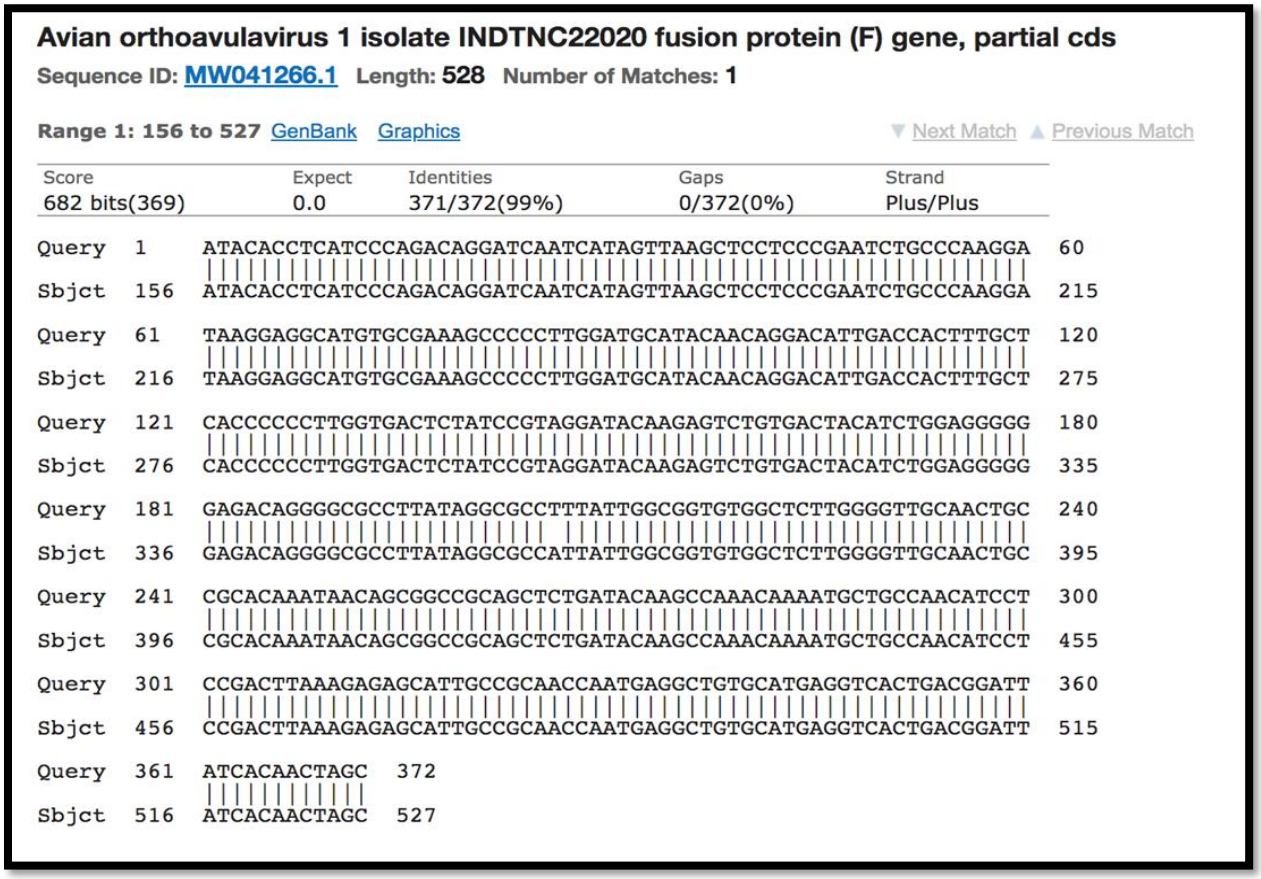
Figure (1): Avian orthovulavirus 1 isolate ByCk/IR/MAME96/2017 fusion protein gene



Avian orthoavulavirus 1 isolate INDTNC22020 fusion protein (F) gene, partial cds

Sequence ID: MW041266.1 **Length:** 528 **Number of Matches:** 1

Range 1: 156 to 527 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
682 bits(369)	0.0	371/372(99%)	0/372(0%)	Plus/Plus

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Query  1    ATACACCTCATCCCAGACAGGATCAATCATAGTTAAGCTCCTCCCGAATCTGCCCAAGGA  60
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Sbjct  156  ATACACCTCATCCCAGACAGGATCAATCATAGTTAAGCTCCTCCCGAATCTGCCCAAGGA  215

Query  61   TAAGGAGGCATGTGCGAAAGCCCCCTTGGATGCATACAACAGGACATTGACCACTTTGCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  TAAGGAGGCATGTGCGAAAGCCCCCTTGGATGCATACAACAGGACATTGACCACTTTGCT  275

Query  121  CACCCCCCTTGGTGACTCTATCCGTAGGATACAAGAGTCTGTGACTACATCTGGAGGGGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  CACCCCCCTTGGTGACTCTATCCGTAGGATACAAGAGTCTGTGACTACATCTGGAGGGGG  335

Query  181  GAGACAGGGGCGCCTTATAGGCGCCTTTATTGGCGGTGTGGCTCTTGGGGTTGCAACTGC  240
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  336  GAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTGGCTCTTGGGGTTGCAACTGC  395

Query  241  CGCACAAATAACAGCGGCCGCAGCTCTGATACAAGCCAAACAAAATGCTGCCAACATCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396  CGCACAAATAACAGCGGCCGCAGCTCTGATACAAGCCAAACAAAATGCTGCCAACATCCT  455

Query  301  CCGACTTAAAGAGAGCATTGCCGCAACCAATGAGGCTGTGCATGAGGTCACTGACGGATT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  456  CCGACTTAAAGAGAGCATTGCCGCAACCAATGAGGCTGTGCATGAGGTCACTGACGGATT  515

Query  361  ATCACAACTAGC  372
            ||||||||||||
Sbjct  516  ATCACAACTAGC  527
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Figure (2): Avian orthovulavirus 1 isolate INDTNC22020 fusion protein gene

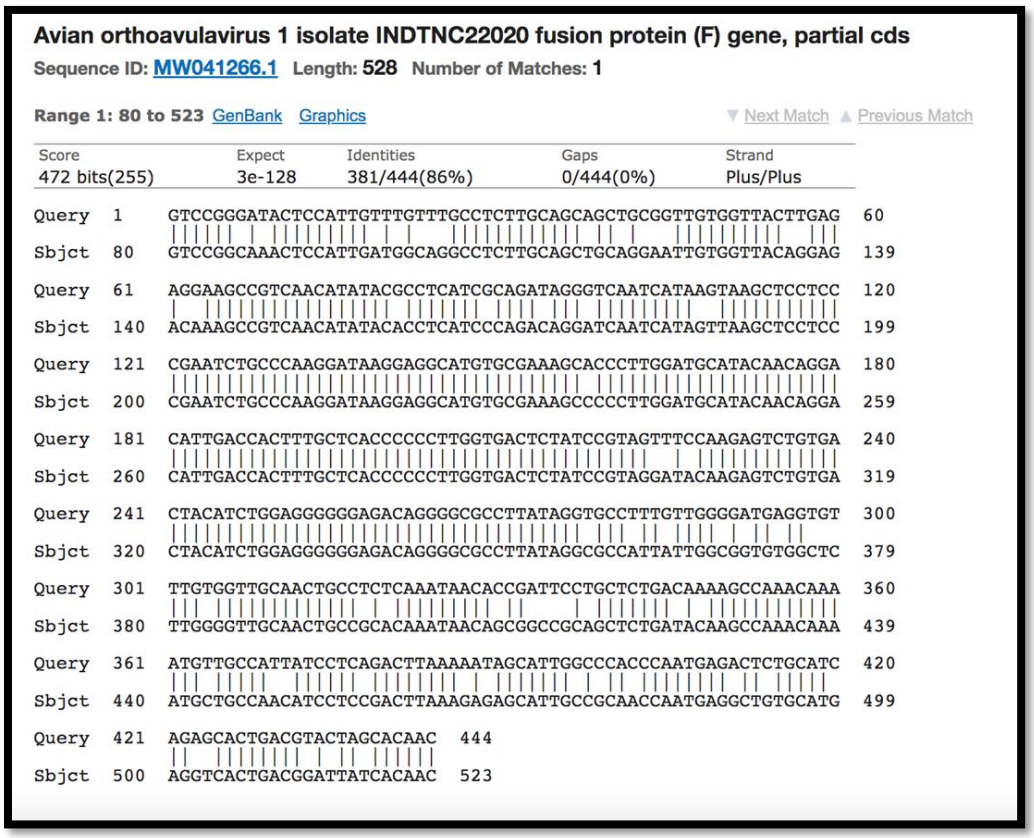

Figure (3): Avian orthovulavirus 1 isolate INDTNC22020 fusion protein gene

Based on the phylogenetic analysis performed in this study, rooted phylogenetic tree (Figure 4) of the nucleotide sequences of the fusion protein gene of Newcastle disease virus strains. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.20497769 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 61 nucleotide sequences. Evolutionary analyses were conducted in MEGA7. Isolates analyzed in this study are marked with filled circle, whereas those reported earlier from the Iraq are marked with empty circles.

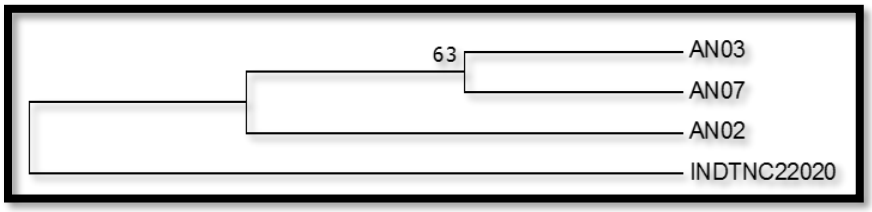

Figure (4): Evolutionary relationships of three isolated Iraqi strains

Discussion

NDV outbreaks in Iraq continue to pose a threat to the country's commercial chicken sector despite the widespread use of vaccinations in the country. It has been postulated that environmental factors, such as secondary immunocompromising infections, might impair the efficiency of vaccinations (AlShammari *et al*., 2020). This could be one explanation for why NDV outbreaks have occurred in populations that have been vaccinated. There is a possibility that vaccination failure and the subsequent epidemic of NDV was caused by inadequate vaccination procedures as well as insufficient levels of flock immunity. Madadgar et al. (2013) proposed three hypotheses to explain the sudden appearance of highly pathogenic NDV. They are as follows: (1) the virus had always been in poultry but was unnoticed until the advent of commercial poultry industries; (2) the virulent virus was enzootic in another species where the less sensitive host revealed the less severe disease; and (3)

the highly pathogenic virus arose from a virus of low virulence by mutation. The first hypothesis proposes that the virus had always been the second theory, on the other hand, has recently emerged as the consensus across communities everywhere. During the panzootic that occurred in the 1970s and 1973, it was discovered that the virus was transferred to certain regions by the migration of captive caged birds, particularly psittacine species, which exhibit some resistance to the viruses that are virulent for chickens. This was discovered during the period of time when the virus was circulating. In spite of the fact that virulent NDV has been discovered in captive caged birds on several times (Madadgar *et al*., 2013), it has been theorized that this is as a result of interaction with livestock that is sick. In a similar vein, there are few reports of wild bird reservoirs of virulent NDV, with the exception of North American cormorants and perhaps pigeons. These two species are the only two known to harbor the virus. The third hypothesis, which posits that virulent viruses evolve as a consequence of mutation from viruses of intermediate virulence, has been supported by research on the viruses that were responsible for virulent NDV outbreaks in Ireland in 1990. These viruses were shown to have antigenic and genetic characteristics with other NDVs, but they were found to be antigenically and genetically distinct from all other NDVs (Sun *et al*., 2022). Diagnostic assays based on different areas of the virus have been devised when F gene based diagnostic primers failed to identify NDV (Miller *et al*., 2010). Last but not least, there is no way to confirm whether or not a flock has been vaccinated since the owner must disclose this information to authorities. Neighbor-Joining was used to estimate the evolutionary history (Saitou and Nei, 1987). The taxonomic lineage is assumed to follow the tree of life derived using the bootstrap method (500 replicates) (Felsenstein, 1985). When a partition is repeated in less than half of the bootstrap replicates, the relevant branch is pruned. In the bootstrap test (500 repetitions), the proportion of trees in which the connected isolates grouped together is shown next to the branches (Felsenstein, 1985). The evolutionary distances are expressed as the average number of base substitutions per site and were calculated using the Jukes-Cantor technique (Jukes and Cantor, 1969). Four different nucleotide sequences were used in the study. The first, second, third, and non-coding locations of the codon table were all included. Blank and empty spots have been filled up completely. The completed dataset had a total of 316 locations. The program MEGA7 was used for the evolutionary analyses (Kumar *et al*., 2016).

Conclusion

Virulent strain-specific motifs were found in the majority of circulating strains, as shown by partial sequencing of the F gene cleavage site.

References

Al-Shammari, A. M., Hamad, M. A., AL-Mudhafar, M. A., Raad, K., & Ahmed, A. (2020). Clinical, molecular and cytopathological characterization of a Newcastle disease virus from an outbreak in Baghdad, Iraq. Veterinary Medicine and Science, 6(3), 477-484.

Bergfeld, J. (2017). The molecular basis of the pathogenicity of Newcastle disease virus in chickens.

Birhan, M., Birhan, M., Tesfaye, S., & Tariku, A. (2019). Detection of antibodies against Newcastle and infectious bursal disease on chicken in north Gondar zone, Ethiopia. Online Journal of Animal and Feed Research, 9(2), 51-58.

El-Tholoth, M., Branavan, M., Naveenathayalan, A., & Balachandran, W. (2019). Recombinase polymerase amplification–nucleic acid lateral flow immunoassays for Newcastle disease virus and infectious bronchitis virus detection. Molecular Biology Reports, 46(6), 6391-6397.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Jukes T.H. and Cantor C.R. (1969). Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, pp. 21-132, Academic Press, New York.

Kumar S., Stecher G., and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.

Madadgar, O., Karimi, V., Nazaktabar, A., Kazemimanesh, M., Ghafari, M. M., Azimi Dezfouli, S. M., & Hojjati, P. (2013). A study of Newcastle disease virus obtained from exotic caged birds in Tehran between 2009 and 2010. Avian Pathology, 42(1), 27-31.

Miller, P. J., Decanini, E. L., & Afonso, C. L. (2010). Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infection, genetics and evolution, 10(1), 26-35.

Moharam, I., Razik, A. A. E., Sultan, H., Ghezlan, M., Meseko, C., Franzke, K., & Grund, C. (2019). Investigation of suspected Newcastle disease (ND) outbreaks in Egypt uncovers a high virus velogenic ND virus burden in small-scale holdings and the presence of multiple pathogens. Avian Pathology, 48(5), 406-415.

Mphuthi, M. B. N. (2018). Development of a real-time PCR assay to detect the fusion gene of the D26 strain of a commercial avian avulavirus 1 (Doctoral dissertation, University of Pretoria).

Naf'an, M. K., Kurniasih, K., Untari, T., & Prakoso, Y. A. (2020). Development of a coagglutination kit as a rapid test for diagnosing Newcastle disease in poultry. Veterinary World, 13(8), 1719.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sun, J., Ai, H., Chen, L., Li, L., Shi, Q., Liu, T., & Liu, S. (2022). Surveillance of Class I Newcastle Disease Virus at Live Bird Markets in China and Identification of Variants with Increased Virulence and Replication Capacity. Journal of Virology, e00241-22.

Susta, L., Diel, D. G., Courtney, S., Cardenas-Garcia, S., Sundick, R. S., Miller, P. J., & Afonso, C. L. (2015). Expression of chicken interleukin-2 by a highly virulent strain of Newcastle disease virus leads to decreased systemic viral load but does not significantly affect mortality in chickens. Virology Journal, 12(1), 1-17.

Tian, K. Y., Guo, H. F., Li, N., Zhang, Y. H., Wang, Z., Wang, B.,... & Zhao, J. (2020). Protection of chickens against hepatitis-hydropericardium syndrome and Newcastle disease with a recombinant Newcastle disease virus vaccine expressing the fowl adenovirus serotype 4 fiber-2 protein. Vaccine, 38(8), 1989-1997.

Tips, H. I. (2017). A Comparative Study of the Serological Status for Newcastle Disease in Local Chickens of Live Bird Markets and Households within Nsukka, Enugu State of Nigeria.

Worku, T., Dandecha, M., Shegu, D., Aliy, A., & Negessu, D. (2022). Isolation and Molecular Detection of Newcastle Disease Virus from Field Outbreaks in Chickens in Central Ethiopia. Veterinary Medicine: Research and Reports, 13, 65.