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Laboratory techniques for isolation, identification and molecular detection of Newcastle disease virus: A systematic review

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Abstract
Newcastle disease virus is a RNA virus that is responsible for causing Newcastle disease, which primarily affects various bird species. The virus has the potential to spread to animals since it can cause conjunctivitis in people who work with poultry. The disease has a significant negative economic impact on the world's poultry production. Cell culture and chicken embryonated eggs are utilized in the viral isolation process. The virus can be identified from blood or allantoic fluids using a variety of techniques, including hemagglutination, hemagglutination inhibition, enzyme-linked immunosorbent assay, and neutralization testing. The mean death time, intravenous, and intracerebral pathogenicity indices can be used to identify viral pathotypes. Reverse transcription-polymerase chain reaction, gene sequencing, and phylogenetic tree analysis are contemporary approaches used to identify and characterize the virus genotypes at the molecular level. In conclusion, this review describes how to isolate and identify the virus that causes Newcastle disease, as well as how to check for the virus's molecular existence, identify its pathotypes, and analyze its genotype. Therefore, using embryonated chicken eggs is generally advised for virus isolation, and further serological and molecular testing should be performed to establish the presence of the virus in the allantoic fluid.

Keywords: Embryonated Chicken Egg, identification, isolation, Newcastle disease virus, RT-PCR, serological tests

Introduction
The global poultry business has experienced numerous socioeconomic crises brought on by various infectious diseases. Newcastle disease (ND), for example, offers a significant economic challenge to the global chicken sector. Newcastle disease was named after the English town of Newcastle, where it was originally discovered in 1927 (Pal, 2007) [31]. It is a fatal disease that kills poultry and results in annual losses of millions of dollars around the world (Susta *et al*., 2010; Waheed *et al*., 2013; Yune and Abdela, 2017) [43, 48, 50]. Newcastle disease virus (NDV) has been reported to affect about 250 different avian species (Suarez *et al*., 2013; Smietanka *et al*., 2014; Ganara *et al*., 2014) [41, 40, 18]. The disease is most prevalent in chickens, turkeys, ducks, and pigeons, with chickens being the most severely affected avian species (Razaeianzadah *et al*., 2011; Zhang *et al*., 2011) [37, 51].
Experimentally, the infection has been produced in bat, ferret, hamster, mouse, monkey, and sheep (Pal, 2007) [31]. A number of domestic and wild bird species are affected by this highly contagious avian disease, which can also infect humans, particularly those who work with infected poultry (Pal and Dube, 1982; Pal, 2007; Pal *et al*., 2013) [31, 32, 33]. In addition to fever, chills, and headache, the affected individuals also experience conjunctivitis, subconjunctival haemorrhage, corneal subpunctate lesion, and preauricular lymphadenitis (Pal, 2007) [31].
Newcastle disease the disease of poultry with virulent strains of NDV (APMV-1), a single-stranded non-segmented RNA virus. It should be notified to OIE and is endemic in countries in Asia and Africa (Brown and Bevis, 2017; Ashraf and Shah, 2014; Afonso *et al*., 2016) [6, 5, 4]. While having a single serotype, all NDV strains differ genetically. According to Diel and co-workers (2012) [13] and Dimitrov and others (2019) [14], there are two classifications of NDVs: class I (avirulent) and class II (which includes low, intermediate, and high virulent strains). Class II contains 18 genotypes **(**Miller S., 2015; Susta *et al*., 2015) [27, 42]. Newcastle disease virus can be diagnosed by inoculating embryonized chicken

chicken eggs or cell lines, performing serological testing (Pal, 2007) [31], or using molecular techniques (Shahzad *et al.*, 2011; Shabbir *et al.*, 2012) [39, 38] .

The most effective way to propagate the virus is by injecting it into the allantoic cavity of an embryonated egg (Zhao *et al.*, 2012) [53]. There are ten subtypes of paramyxoviruses (APMV-1 to APMV-10) have been isolated from avian species (Miller *et al.*, 2010, Waheed *et al.*, 2013) [28, 48]. Newcastle disease, a prevalent viral disease in poultry, has a significant negative impact on the economy. Therefore, the objective of this paper is to present an overview on the techniques used to isolate, identify or detect Newcastle disease virus at molecular level.

Background of Newcastle Disease Virus

Newcastle disease was diagnosed in a patient in Indonesia and England in 1926 and 1927, respectively. Since then ND is endemic in many nations, the OIE has classified it as a reportable disease (OIE, 2018) [30]. The Newcastle disease virus is classified as belonging to the Mononegavirales (order), Paramyxoviridae (family), Paramyxovirinae (subfamily), Avulavirus (genus), and serotype 1 (APMV-1) (Cattolli *et al.*, 2011; Waheed *et al.*, 2013) [8, 48].

Newcastle disease virus virions are filamentous and slightly spherical in shapeas indicated in Figure 1 (Catroxo *et al*., 2011) [9]. The genome is 15.2 kb in length and codes for 2 non-structural and 6 structural proteins (Choi *et al.*, 2010; Zhang *et al*., 2012; Cao *et al.*, 2013) [11, 52, 7]. According to Linda and others (2011) [26], Qiu and co-workers (2011) [36], and Al Habeeb and c-investigators (2013) [3], W and V are non-structural whereas NP, L, F, HN, M, and Pare are structural. The three types of Newcastle disease virus strains are the virulent (velogenic), moderately virulent (mesogenic), and nonvirulent (lentogenic) strains. To provide protection against virulent strains, a live vaccination can be created from the nonvirulent LaSota strain (Mansour *et al*., 2016) [25].

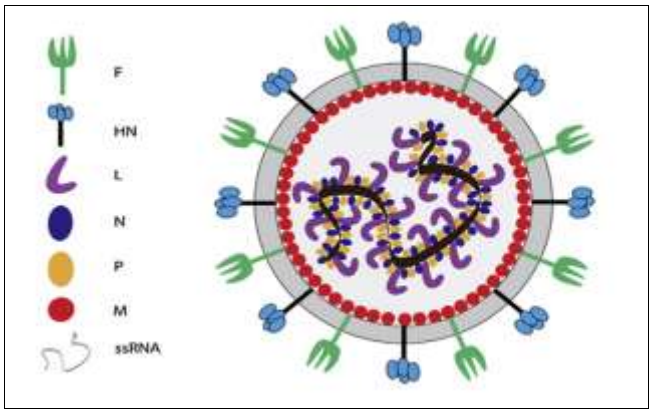

Fig 1: Morphological structure of Newcastle disease virus (Source: Ganar, 2014) [18].

Isolation of Newcastle Disease Virus

Newcastle disease virus can be isolated from or identified in samples from live or dead birds. Internal organs such the spleen, lung, kidneys, gut, caecal tonsils, liver, and heart tissues can be used from dead birds, along with the brain and oro-nasal swab. Tracheal, oropharyngeal, and cloacal swabs from live birds may be used. Samples should be transported in viral transporting media, isotonic phosphate buffered saline (PBS), which contains antibiotics and has a pH of 7.0-7.4 (OIE, 2018) [30].

Inoculation of a virus into an embryonated egg

Sample (organs and/or swabs) centrifuged at 4000 rpm for 5 minutes, antibiotics added to the supernatants, and incubated for 1-2 hours at room temperature. Then, as shown in Figure 2, 0.2 mL of supernatant was inoculated into the allantoic cavity of chicken eggs that had been specifically pathogen-free (SPF) embryonated and aged between 9 and 11 days. The control embryonated egg should be incubated at 37°C for 4–7 days while being monitored daily. It should also be infected with sterile normal saline (OIE, 2012) [29].

Embryos died within 24 h post-infection are death due to non-specific reasons and that died after 24 h or survived until the end of incubation are refrigerated at 4 °C overnight and allantoic fluids should be examined by hemagglutination (HA) test. Positive HA allantoic fluid should be collected and stored at -80 °C (OIE, 2012; Alazawya and Al Ajeli, 2020) [29, 2]. The embryo death post inoculation is due to the virus induced agglutination of red blood cells. The chick embryos supported the extensive replication of the virus so that is the possible reason for widely use it for NDV growth (Hemmatzadeh and Kazemimanesh, 2017; Mahboob *et al.*, 2020) [19, 24].

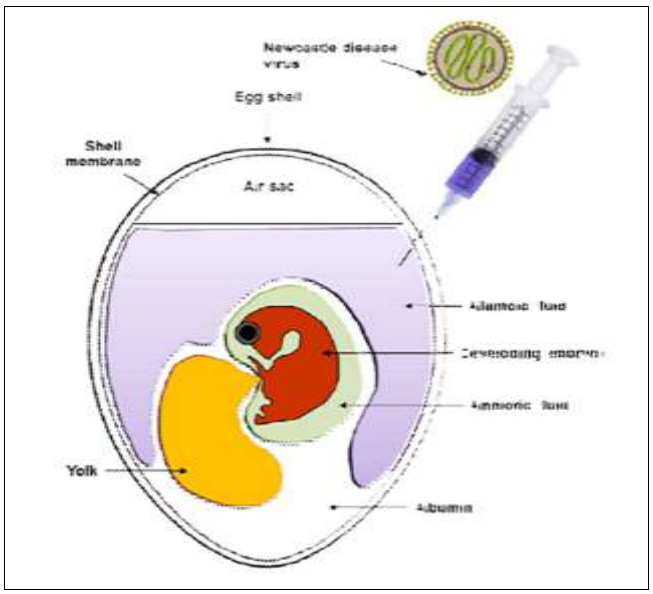

Fig 2. Newcastle disease virus inoculation intoembryonated chicken egg (Source: Mansour *et al.*, 2016) [25].

Cell culture

Newcastle disease virus can replicate in a variety of avian and non-avian cell types, but it most frequently favors chicken embryonic kidney (CEK), chicken embryonic liver (CEL), chicken embryonic fibroblasts (CEF), African green monkey kidney (Vero) cells, and chicken embryo-related (CER) cells (Terragino and Capoa, 2009) [45]. The best cells for NDV replication are primary and/or continuous avian cells, such CEF DF-1 cells. To increase the chances of viral retrieval for low virulent isolates, trypsin is added to the medium, and virus replication is followed with cytotoxic effects (CPE) (OIE, 2018) [30]. Small plaques, rounding of cells, foci and syncytia development, cell death, rupture of cell monolayers, and detachment from culture plate are some of the CPE seen in inoculation samples during viral isolation (Maqbool *et al*., 2017 [26,].

Identification of Newcastle Disease Virus

The presence of antibodies in birds can be determined using a range of serological techniques, including the neutralization test, enzyme linked immunosorbent assays (ELISA), and hemagglutination inhibition (HI). The virus that causes Newcastle disease serves as an antigen in serological assays to measure antibody levels. The most widely used test to identify antiviral antibodies is hemagglutination inhibition, and post-vaccination antibody levels are typically assessed using ELISA (Pal, 2007) [31]. Reverse-transcriptase polymerase chain (RT-PCR) is also used to confirm NDV from isolates or samples, in addition to the aforementioned serological assays. However, as vaccination is universal, serological testing are mostly used to monitor antibody response following vaccination rather than for the diagnosis and surveillance of ND (Chaka *et al*., 2013; OIE, 2018) [10, 30].

Hemagglutination test (HA)

The measurement of virus particles extracted from cell culture or allantoic fluid supernatant using this indirect technique is very common. NDV contains proteins that can attach to and agglutinate red blood cells (RBCs), which is the basis for this assay. For this test, chicken blood was obtained in tubes coated with an anticoagulant, centrifuged, the serum was removed, and RBCs were then filtered using Alsever's buffer to 5% pure RBCs after numerous centrifugations (Mansour *et al*., 2016) [25].

In this test, 0.025 ml of PBS is distributed among the wells, and an equal amount of virus is added to the first well before a series of dilutions are applied to the other wells. Then, carefully mix in an additional 0.025 ml of PBS and an equal volume of 1% chicken RBCs into each well. It is recommended that the test and control RBCs (in the different button) settle for 40 minutes at room temperature. HA determined by tilting the plate, it is possible to see whether there is tear-shaped RBC streaming (OIE, 2018) [30]. Slide hemagglutination (HA) testing can also be used to determine whether a hemagglutinating virus is present in allantoic fluid. For RT-PCR and sequencing analysis, allantoic fluids evaluated by the slide HA test and NDV-positive are collected and stored at -70 °C or -80 °C (OIE, 2012) [29].

Hemagglutination inhibition (HI)

Samples that tested positive for HA should be checked for the presence of NDV using a recognized NDV antiserum (OIE, 2012) [29]. Each well of the plate is filled with 0.025 ml of PBS, then an equivalent volume of serum is added. The serum is then diluted twice through the plate. Each well needs to have 0.025 ml of the HA Units virus or antigen added before it stays at room temperature for 30 minutes. Then each well received 0.025 ml of 1% chicken RBCs, which were moderately mixed and left for 40 minutes at room temperature. As a result, agglutination is measured by tilting the plates while taking samples from control wells that indicated inhibition. A negative control serum that does not provide a titer and a positive control serum for which the titer should be within one dilution of the known titer should be used to compare the results' validity (OIE, 2018) [30].

Viral pathotyping

Mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) approaches can all be used in viral patho typing. In MDT, embryonated SPF chicken eggs that were 9 to 11 days old were serially diluted 10 times with NDV isolates and incubated at 37 °C until the embryos died (OIE, 2018) [30]. According to Pedersen (2011) [34] and Putri and co-investigators (2017) [35], if an embryo died within 60 hours, it is velogenic, between 60 and 90 hours, it is mesogenic, and after 90 hours, it is lentogenic.

In the ICPI procedure, infective allantoic fluid is diluted at a ratio of 1:10 in sterile isotonic saline and 0.05 mL of the dilution is injected intracerebrally using a sterile syringe into the caudal region of day-old chick brains hatched from SPF flock eggs. Every 24 hours for 8 days, the chick should be examined, and if healthy, sick, or dead, it should be given a score of 0, 1, or 2. The ICPI is determined as the mean score for each bird seen during an eight-day period; the value is near to 2 for virulent strains and close to 0 for lentigogenic or asymptomatic strains of enteric bacteria. ICPI values range from 1.3 to 2.0 velogenic, between 0.7 and 1.3 mesogenic, and less than 0.7 lentogenic NDV (OIE, 2012; Hossain *et al.*, 2017) [29, 20].

In the IVPI, 6-week-old SPF chickens get an intravenous injection of 0.1 mL allantoic fluid containing virus diluted at a ratio of 1:10 in sterile isotonic saline. The chickens are then observed after 10 days and scored 0, 1, 2, or 3 depending on whether they are healthy, ill, paralyzed, or dead. Therefore, the IVPI is the average score for each bird observed over a 10-day period. If lentogenic and some mesogenic strains are present, the value is close to 0, while virulent strains have a value closer to 3 (Hossain *et al*., 2017; OIE, 2018) [20, 30].

Enzyme linked immunosorbent assay (ELISA)

A variety of ELISA kits, including indirect ELISA, sandwich ELISA, and competitive ELISA employing monoclonal antibodies (MAbs), are available to detect NDV antibodies. These kits should be used in accordance with the manufacturer's instructions and intended usage. The HN protein is the only antigen that the HI test can detect antibodies to, in contrast to ELISAs, which measure antibodies to several antigens (OIE, 2018) [30]. At room temperature, serum samples are used for the NDV ELISA. The serum should be distributed into identical wells and incubated for 30 min after being diluted at a ratio of 1:500 in Dulbecco's phosphate buffered saline (DPBS), along with the negative and positive controls. Following a third wash, conjugate should be added, and the plates should then be incubated for 30 minutes. Wells were once more rinsed three times, substrate solution is added, and incubation was given for 30 minutes. After that, plates read and stop solution is added right away (Chumbee *et al*., 2017) [12].

Viral neutralization test (VNT)

Hemagglutination Inhibition assay and ELISA are used to measure NDV-specific antibodies but not neutralize antibodies (nAbs) against NDV. The neutralization test (NT) can be used for this. Traditional NT is time-consuming, tedious, and subject to operator biases. However, in recent years, genetically altered viruses that express the green fluorescent protein (GFP) or the enhanced GFP (eGFP) have been employed for eGFP-based NT (eGFP-NT) to quickly assess the titers of antibodies that neutralize viruses or the effectiveness of antiviral agents. In conventional NT, virus-serum is added to cell cultures like

CEF DF-1 cells, they are cultured for 4 days at 37 °C with 5% CO2, and then they are rinsed with DPBS. After that, cells are fixed and stained for 15 minutes at room temperature with a solution of 0.2% crystal violet and 3.2% paraformaldehyde. The reciprocal of the highest dilutions is used to calculate NDV nAb titers (Chumbee *et al*., 2017) [12]. Using mixes of virus-serum, DF-1 cells are infected with IneGFP-NT, incubated for 48 hours, and then examined under a fluorescence microscope. The reciprocal of the highest dilutions that did not express eGFP is then used to calculate NDV nAb titers. As a result, the wells with luminous foci are seen favorably (Yager and Moore, 2015; Chumbe *et al*., 2017) [49, 12].

Molecular Detection of Newcastle Disease Virus

Although viral isolation is regarded as the gold standard for diagnosing NDV, molecular methods such as RT-PCR and nucleotide sequencing analysis are critical for diagnosing viruses (Shahzad *et al*., 2011; Shabbir *et al*., 2012) [39, 38].

Viral RNA extraction

There are a number of RNA extraction commercial kits and reagants that can vary in performance. TRIZOL® LS, Qiagen® Rneasy Mini Kit, Mag MAXTM-96 AI/ND Viral RNA Isolation Kit, and ISOGEN II (NIPPON GENE) reagents are a few of the commercially available RNA extraction kits and reagents (Dimitrov *et al*., 2014; Tran *et al*., 2020) [14, 47]. In order to enable amplification of a portion of the gene sequence, complementary deoxyribonucleic acid (cDNA) synthesis kit are used to convert viral RNA to cDNA. The cDNA produced from the synthesizing step is then included into PCR techniques with the necessary gene specific pair of primers (Esmaelizad *et al*., 2012) [17].

Polymerase Chain Reaction using Reverse Transcription (RT-PCR)

Primers are designed using conserved NDVgene sequences from different isolates that were downloaded from GenBank on the National Center for Biotechnology Information (NCBI) website. Reverse transcription of the gene segment results in cDNA, which is then amplified using a thermocycler PCR equipment. The NDV genes' forward and reverse primer sequences are utilized, respectively, for forward and reverse direction amplifications. Most commonly utilized for molecular detection and characterisation of NDV are the M gene, the highly conserved gene segment, the F gene, which is a virulent determinant and the HN gene (Abah *et al.*, 2016; Elmardi *et al*., 2016) [1, 16].

RT-PCR is performed by reversetranscription (incubationat 50 °C for 20 min with reverse transcriptase enzyme to synthesize cDNA), initial denaturation (incubation at 95 °C for 15 min to stop reverse transcription),denaturation of cDNA (incubation at 94 °C for 45 sec for primer attachment to each single strands), annealing (incubation at 60 °C for 45 sec for attachment of primers and deoxynucleotide triphosphates (the building blocks) and extension (incubation at 72 °C for 45 sec for extending the fragment).The denaturation, annealing and extension should be repeated for 40 cycles with a final extension at72 °C for 5 min. The PCR products should be preserved at 4 °C (Abah *et al.*, 2016; Elmardi *et al*., 2016) [1, 16].

Gene sequencing and phylogenetic tree analysis: The amplified DNA fragments from the PCR product, which were purified using a PCR purification kit, are sequenced using an applied biosystems (ABI) sequencing machine, checked for errors, and edited for homology searches in an online database of nucleotides (NCBI GenBank) with the aid of software called basic local alignment search tool (BLAST). A phylogenetic tree can be created by integrating the obtained sequences into the molecular evolutionary genetic analysis (MEGA) software when the GenBank database issues accession numbers or IDs following NCBI examination of the sequences (Kumar *et al*., 2016) [22] .

Since the F protein defines the virulence of the strains, it was the protein used by the majority of researchers to analyze NDV; however, little information is known about the HN protein (Esmaelizad *et al*., 2012) [17]. However, by performing a phylogenetic analysis on HN sequences and comparing them to HN sequences of NDV strains from various studies, it is possible to distinguish the genetic variety of NDV isolates (Kiani *et al*., 2020) [21]. Thampaisan and colleagues (2017) [46] claimed that next-generation sequencing (NGS) can produce the full-length viral genome's nucleotide sequence. The maximum-likelihood method can be used to build a phylogenetic tree using MEGA software for genotype identification, and the nucleotide and deduced amino acid sequences of proteins can be aligned, analyzed, and compared with sequences available in Gen Bank and identified by BLAST (Tamura *et al*., 2013) [44].

Conclusion

Newcastle disease virus is an enveloped, single stranded RNA virus having a great effect on poultry production. This review states that the virus can be isolated from samples from both cell cultures and allantoic of chicken egg embryos. Newcastle disease virus can be identified by serological techniques, such ELISA, viral neutralization, hemagglutination assays, hemagglutination inhibition, and pathogenecity indices. It is advised to identify virus pathotypes using the mean death time, intracerebral pathogenecity index, and intravenous pathogenecity index. Molecular detection of the virus, such as viral RNA extraction for RT-PCR, nucleotide sequences and analysis based phylogenic tree are mostly using for isolates of samples in different countries and are the modern methods important in determination of genotypes. As the gold standard method, viral isolation on SPF embryonated chicken eggs should be verified with HA, HI, and RT-PCR to ensure accurate results. The RT-PCR primer is recommended based on the project's goals, such as viral detection, characterization, and/or pathogenicity.

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Author's Contribution

All the authors contributed equally,and approved the final version of the manuscript for its submission

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