

Molecular Diagnosis of Newcastle Disease Virus by RT-PCR and Detection Fusion Gene Using Conventional PCR

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Abstract

Newcastle disease is one of the worst avian illnesses, it was responsible for 32.7% of all deaths. The significant mortality rates from Newcastle disease illnesses necessitate that, they be given top care.

The main purpose of the current study was Molecular diagnosis of Newcastle disease virus by RT-PCR and detection fusion gene using conventional PCR.

With the discovery of Newcastle disease symptoms in 20 flocks, researchers tested 70 random samples and 40 samples as healthy control from all around the governorates of Babylon and Al-Najaf (loss of appetite, coughing, gasping, nasal discharge, watery eyes, bright green diarrhea and nervous signs such as paralysis and convulsions). In the beginning, an NDV kit test was utilized to quickly verify the infection. The best time to start raising chickens is between day 20 and day 35. The flocks were protected against NDV using LaSota vaccinations. The samples were collected between October 2021 and January 2022. To verify the tentative diagnosis based on clinical and pathological signs, 5 ml of blood were drawn. Each blood sample resulted in two separate test tubes (2.5ml putted in EDTA tube, to obtain of whole blood, and 2.5ml putted in gully tube to obtain serum). All samples were stored at -20C until analysis. Field examination was performed by an NDV rapid kit where it showed positive results for 63(90%) samples out of 70 samples were detected as ND according to clinical symptoms, out of 63 samples, 40(63.4%) samples give positive inhibition. Haemagglutination inhibition test were determination on 63 clinical samples that showed positive results of NDV by immunochromatography Rapid diagnostic test. The results showed that, out of 63 samples, 40(63.4%) samples give positive inhibition. There were significant difference between mean antibody titer of infected birds and healthy control group, the mean of AB titer was decrease (13.15±1.94) more than control (467.2±48.83). Results from an RT-PCR test revealed that, only 12(30%) of 40 isolates were positive. Seven of the twelve NDV isolates underwent RT-PCR to identify the F gene, and the findings indicated that, two of the samples provided a strong band, one gave a light band, one gave a weak band, and three did not exhibit any band of F gene.

It was conclude that, in order to effectively develop a control strategy against the illness, rapid test detection and identification of the viruses are essential. As a result, the RT-PCR molecular technique may be used for quick and confirmatory detection of NDV from any kind of ND epidemic. The RT-PCR assay also had a higher detection rate of NDV than the HI test. Since this is the case, introducing the molecular approach (RT-PCR) for quick and confirmatory detection of NDV from any kind of ND outbreak at the field level of Iraq is feasible.

Keywords: Fusion Gene, Newcastle Disease, Real Time-PCR.

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INTRODUCTION

If rural development programs include poultry, it might be a useful tool for swiftly filling up poverty gaps. It reproduces quickly and has a short generation time. It reproduces quickly, requires little care from the farmer, and can have its productivity increased more quickly and readily than most other animals. Many countries provide large-scale, small-scale, and commercial poultry industries to its scavengers. Chickens from each of the three production methods are unique in terms of their need for inputs and the qualities they produce. Each has the potential to help address the economic and social issues plaguing a variety of communities

throughout time (Pius *et al.*, 2021). Newcastle disease is one of the worst avian illnesses (ND). Funsho-Sanni *et al.*, (2022) found that, ND was responsible for 32.7% of all deaths. The significant mortality rates from ND illnesses necessitate that they be given top care. Many books and articles have been written on the frequency of ND antibodies in chickens. Despite the worrisome rise in ND seroprevalence throughout Iraq and many other countries, no studies have been conducted to estimate mortality and morbidity rates or to identify risk variables via follow-up or sero-epidemiological analysis (AlShammari *et al.*, 2020). Highly infectious and fatal to poultry, Newcastle disease (ND) is caused by virulent

strains of the Newcastle disease virus (NDV), also known as avian paramyxovirus type 1. (APMV-1). The virus belongs to the genus Avulavirus, subfamily Paramyxoviridae (Harazem *et al.*, 2019). In 1926, the first cases of Newcastle disease were reported in Java, Indonesia, and Newcastle upon Tyne, England. As a consequence of its widespread spread, the poultry industry has suffered significant financial losses. The most sensitive animal is the chicken, which may suffer from minor infections with no outward symptoms to fatal ones (Ashraf *et al.*, 2018). As of yet, there is no confirmed cure or strategy for completely eliminating Newcastle disease. Biosecurity, importing chickens from flocks free of illness, and lifelong immunization are all methods of prevention. Vaccines against ND have traditionally been made by cultivating viral strains in chicken embryos. Allantoic fluid is collected for its NDV content and then used to produce a vaccine (Campbell *et al.*, 2018). However, this conventional approach has significant problems, such as a lack of quality control, a high labor intensity, a lengthy incubation period, a great quantity of particular pathogen-free eggs, and a large space in which to incubate the eggs. However, due to the process's slowness and complexity, huge strategic stockpiles must be maintained to react to epidemics (Suarez *et al.*, 2020). Rapid, sensitive, and cost-effective alternatives to traditional procedures may be found in genetic-based assays, which are most typically used for routine diagnosis. Fusion (F) or haemagglutinin-neuraminidase (HN) genes exhibit large variations, however RT-PCR that zeroes in on a highly conserved gene may account for these differences. After being pretreated, they may be utilized on clinical samples for viral RNA extraction and testing in bulk (Bello *et al.*, 2018). The next step after a positive real-time RT-PCR detection utilizing a highly sensitive and comprehensive screening strategy is to sequence the F gene to ascertain the virus's pathogenicity (at the F gene proteolytic cleavage site) and genotype. Tests used should have shown sensitivity for detecting either endemic or emerging viruses in the area where they are used (Butt, 2019). It is suggested that a real-time RT-PCR screening test be used to detect both virulent and avirulent APMV-1. Whether a test comes back positive, you may do further specific testing to see if the virus has a cleavage site that works with NDV's fusion cleavage. In order to detect all virulent genotypes circulating in a nation or area, gene sequencing is favored over RT-PCR because of the diversity of the fusion cleavage site (Bello *et al.*, 2018). Direct sequence of the fusion cleavage site or virus isolation and classical analysis may be necessary for outbreaks involving birds showing symptoms compatible with Newcastle disease virus that test positive by the real-time RT-PCR screening test but test negative by the virulent fusion RT-PCR tests (Pandarangga, 2021).

MATERIALS AND METHODS

Throughout numerous places in the governorates of Babylon and Al-Najaf, 70 random samples and 40 samples as healthy control were tested because 20 flocks showed clinical and

pathological indicators of Newcastle disease (loss of appetite, coughing, gasping, nasal discharge, watery eyes, bright green diarrhea and nervous signs such as paralysis and convulsions). At initially, an NDV kit test was utilized to quickly verify the infection. Ages 20–35 days are considered the sweet spot for chickens. When protecting their flocks from NDV, they employed LaSota immunization. The samples were collected from late 2021 through early 2022. Five milliliters (5 ml) of blood were drawn to verify the diagnosis reached via clinical and pathological signs of illness. Each blood sample resulted in two separate test tubes (2.5ml putted in EDTA tube, to obtain of whole blood, and 2.5ml putted in gully tube to obtain serum). All samples were stored at -20C for further examination.

Quick Start Kit

After these things were looked at, the diagnosis was verified by a short NDV test performed by a professional (rapid kit). A Bionote Korean Rapid kit was used in the study. To qualitatively identify NDV antigen in serum, you may use a chromatographic immunoassay, such the one provided by the Antigen Quick NDV Antigen Testing Kit. For example, the Anigen Rapid NDV Antigen kit included two letters, T and C, which stood for "test line" and "control line," respectively. Before a number of samples have been submitted, neither the test line nor the control line's output window will be visible. It is sufficient to examine the control line in order to ascertain the correct outcomes of an experiment. A trap and a detector, the highly selective NDV antibodies are at the heart of this test. It's possible that, these antibodies can reliably identify the NDV antigen present in the sample.

Haemagglutination tests

1. Each of the 96 wells on a plastic Ushape-bottomed microtitre plate was filled with 25 l of PBS.
2. The second well contained 25 l of viral suspension (infective allantoic fluid).
3. The viral solution was diluted by a factor of two using 0.025 ml portions and spread out onto a plate.
4. Twenty-five microliters of chicken RBCs diluted to a final concentration of 1% (v/v) were added to each well.
5. The RBCs were settled for approximately 15 minutes at room temperature while the suspension was stirred by tapping the plate lightly.
6. HA was determined by tilting the plate and watching for tear-shaped streaming of the RBCs after the control RBCs had been settled to an unique button.
7. Titrations were read to the maximum dilution that gave full HA (no streaming); 1 HA unit (HAU) was equal to this value, and it was properly computed from the original dilution range.

A drop of the allantoic fluid to be examined was combined with a drop of pooled chicken erythrocytes at a concentration of 10%, and the resulting reaction was seen using a low-magnification microscope or visually.

Haemagglutination Inhibition test

1. Twenty five microlitre of PBS was dispensed into each well of a plastic Ushape bottomed well microtitre plate.
2. Twenty five microlitre of serum was placed into the first well of the plate.
3. Two fold dilutions of 0.025 ml volumes of the serum were made across the plate.
4. Four (HAU) virus/ antigen in 25 microlitres was added to each well and the plate was left for a minimum of 30 minutes at room temperature.
5. 0.025 ml of 1% (v/v) chicken RBCs was added to each well and, after gentle mixing. The RBCs were allowed to settle for about 40 minutes at room temperature, with checking each 5-10 minute.
6. The HI titer was the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination was assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.025 ml PBS only) was considered to show inhibition.
7. The HI titer of the serum was computed for the Beta procedure by multiplying the reciprocal of the serum titer by the number of HA units used in the test.

Viral RNA extraction

Using Viral Gene-spin™ Viral DNA/RNA Extraction Kit).

cDNA synthesis

Using AddScript cDNA Synthesis Kit.

Real-Time PCR assay

The traditional Real-Time PCR method was applied on all of the possible samples. The NDV gene's conserved region was amplified using a single set of targeted primers. The reactions were performed using 2 ng of genomic DNA and a GoTaq® Probe qPCR Master Mix in 0.2 l wells, for a total volume of 20 ml (Promega, USA). Following this, the wells were placed in a thermal cycler (Stratagene, USA) and heated to 95 degrees Celsius for 10 minutes, followed by 40 cycles of 95 degrees Celsius for 15 seconds, followed by 1 minute at 60 degrees Celsius. Over 95% of samples were successfully amplified, with an error rate of less than 1% computed from PCR duplicates.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS version 25. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Student t-test was used to compare means between two groups.

RESULTS

Seventy randomly samples have been investigated in different places of Babylon and Al-Najaf governorates, samples were collected from 20 flocks that showed clinical and pathological signs related to Newcastle disease (loss of appetite, coughing, gasping, nasal discharge, watery eyes, bright green diarrhea and nervous signs such as paralysis and convulsions). The targeted age of the chicken will be 20-35 days. All samples were collected from October 2021 to January 2022. The case history of the fields included in the study was represented in the vaccination program. The methods of administration, type of vaccine strain, and the age of vaccination differed according to the vaccination program for each flock. Newcastle LaSota vaccine was used, it was administrated by different methods include spraying, eye drop or drinking water. Another aspect that could not be overlooked was the administrative side, which was in poor condition in most flocks, as neglecting biosecurity was an important catalyst for disease outbreaks. Table (1) shows the details of each case that was recorded during sample collection. The owners were contacted after sampling to collect detailed mortality information, but in some cases we were unable to contact them.

Table (1): Initial detection of Newcastle disease according to symptoms

Type of Organ	Region	Initial detection of Newcastle disease according to symptoms
Lung	Hillah	22(31.4%)
	Najaf	8(11.4%)
Trachea	Hillah	20(28.6%)
	Najaf	20(28.6%)
Total 70(100%)		

Field examination was performed by an NDV rapid kit where it showed positive results for 63(90%) samples out of 70 samples were detected as ND according to clinical symptoms, the positive results were read by the appearance of the indicative line at the letter (T) and the letter (C) together, in contrast to the negative result that is only at the (C) line as in Figure (1).



Figure (1): immunochromatography rapid diagnostic test a- positive, the indicative line appeared at C and T, b- negative, at C only

Haemagglutination inhibition test were determination on 63 clinical samples that showed positive results of NDV by immunochromatography Rapid diagnostic test. This might be due to the presence of high concentration of free NDV these samples. The anti-NDV hyper immune serum revealed complete inhibition of the haemagglutination unit of each samples. The results showed that, out of 63 samples, 40(63.4%) samples give positive inhibition. Antibody titer of control and HI infected birds were showed in Table (2), Figure (2). There were significant difference between mean antibody titer of infected birds and healthy control group, the mean of AB titer was decrease (13.15 ± 1.94) more than

control (467.2 ± 48.83).

Table (2) AB titer in control and HI infected birds

Groups	No.	Mean \pm SE
Control	40	467.2 ± 48.83
Infected birds	40	13.15 ± 1.94
T test	9.290	
Calculated P value	$<0.001^*$	

* Significant difference at $P < 0.05$

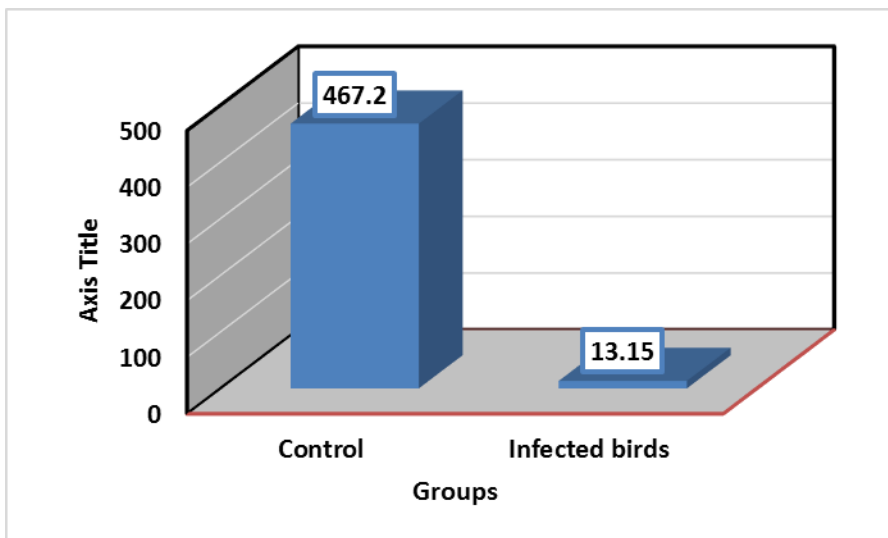


Figure (2): AB titer in control and HI infected birds

In an RT- PCR test, the detection of fluorescent signal aggregation determines the positive response. The number of cycles required to reach the threshold is the number Ct (cycle threshold). The ratio of the Ct value to the amount of target

nucleic acid in the sample is inversely related; The Ct values in the current study ranged from 37-40 cycles. The primers are designed with a broad spectrum including a wide number of virus strains. The reading of the RT-PCR test result was

showed that, only 12(30%) isolates out of 40 isolates showed a positive result as shown in Table (3), Figure (3).

Table (3): RT-qPCR for detection of Newcastle virus (tested samples= 40)

Type of Organ	Region	Newcastle virus detection by (RT-PCR)	Negative isolated
Lung	Hillah	7(17.5%)	8(20%)
	Najaf	-	8(20%)
Trachea	Hillah	4(10%)	6(15%)
	Najaf	1(2.5%)	6(15%)
Total (40)		12(30%)	28(70%)

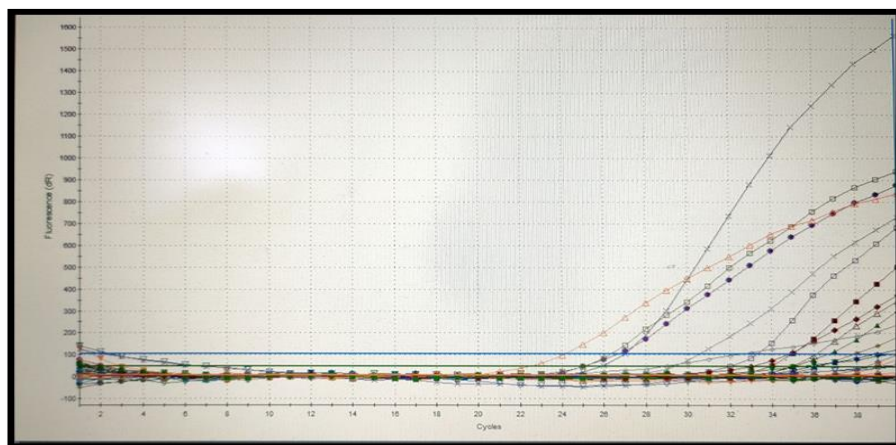


Figure (3): RT-qPCR curve for detection of Newcastle virus

Molecular study of *F* gene was detected in 7 isolates out of 12 NDV isolates by RT-PCR, the results showed that, two isolates were gave strong band, one isolates was gave faint band, one isolate was gave weak band and 3 isolates were not appeared any band of *F* gene, the positive results were detected by the presence of (535 bp) bands when compared with allelic ladder as shown in Table (4), Figure (4).

Table (4): Detection of Fusion gene (*F* gene) by conventional PCR

Sample	Organ	RT-qPCR result	Conventional PCR result
AN02	Lung	Positive	Weak band
AN03	Lung	Positive	Strong band
AN05	Lung	Positive	No band
AN07	Trachea	Positive	Faint band
AN09	Trachea	Positive	Strong band
AN10	Trachea	Positive	No band
AN14	Lung	Positive	No band

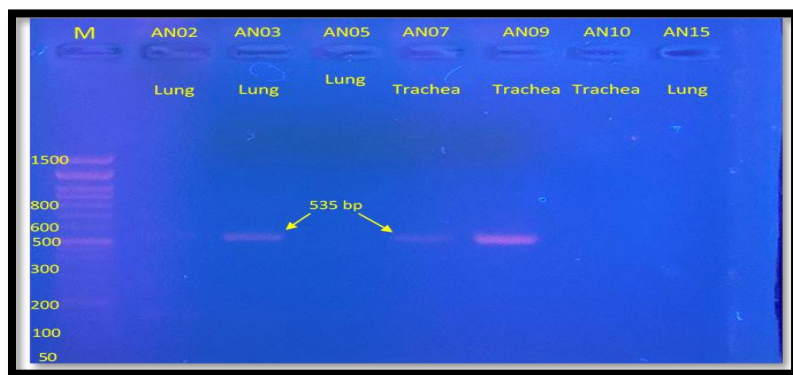


Figure (4): Conventional PCR for identification of gene *F* of Newcastle virus (NDV). The expected PCR band for detection of NDV virus is 535 bp. All seven tested samples were positive for NDV by RT-qPCR assay. The gel was 1.5% and the DNA dye is Red Safe (Intron, Korea). V: 90, Time: 40 minutes. M: DNA ladder

DISCUSSION

Given the severity of the disease's effect on chickens, the great majority of NDV-related poultry references focus on this species. This species has such a broad range of illness presentations that clinical data is further subdivided into pathotypes (Pereira *et al.*, 2022). The virulence of a virus is a major influence in how severely an infection manifests in a human host, although other host-related variables may also play a role. Age, mode of infection, immunological condition, and the presence of other stressors all have a role. Aerosolization of large viral doses seems to primarily affect the upper respiratory tract, and younger animals are more likely to have severe and acute sickness than older animals, and intravenous injection is more likely to elicit neurologic symptoms (Susta, 2011). Despite regular immunization, NDV outbreaks in Iraq represent a persistent danger to the country's commercial chicken industry. Environmental variables, such as secondary immunocompromising diseases, have been hypothesized to reduce vaccination effectiveness (AlShammari *et al.*, 2020), which might explain why NDV outbreaks have occurred in vaccinated populations. Inadequate vaccination methods and low levels of flock immunity may contribute to vaccination failure and subsequent NDV outbreaks. To explain the sudden appearance of highly pathogenic NDV, Madadgar *et al.*, (2013) proposed three hypotheses: (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. As of late, though, the second explanation has become the standard across communities. During the 1970s and 73 panzootic, 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 virulent for chickens. Though virulent NDV has been found in captive caged birds on several occasions (Madadgar *et al.*, 2013), it has been hypothesized that this is due to contact with infected livestock. Similarly, there are inadequate reports of wild bird reservoirs of virulent NDV, with the exception of North American cormorants and probably pigeons. Research on the viruses that caused the virulent NDV outbreaks in Ireland in 1990 supports the third theory, which proposes that the virulent viruses develop as a result of mutation from viruses of moderate virulence. It was discovered that these viruses had antigenic and genetic features with other NDVs, but were antigenically and genetically unique from all other NDVs (Sun *et al.*, 2022). Afonso, (2021) also shown that a low-virulence duck isolate became highly pathogenic for hens after being transmitted through them nine times using the air sac injection procedure and subsequently five times by intracerebral inoculation. Because of this, it seems that virulent NDV isolates that do not infect exotic cage birds may infect industrial poultry and

eventually become epizootic. According to Rehan *et al.*, (2019), 38.63% of NDVs had a favorable outcome. According to the results of the reverse transcriptase polymerase chain reaction, 17 of the clinically ill and deceased hens tested positive for the virus. According to Worku *et al.*, (2022), 54.54 percent of responses about NDVs were favourable. The RT-PCR results from this investigation indicated that out of a total of 40 isolates, only 12 (30%) tested positive. Out of 34 field samples, 26 NDV isolates according to HI were positive for the identification of ND virus genome by RT-PCR, as examined by Hasan *et al.*, (2010), who used a molecular detection approach including RT-PCR. Selim *et al.*, (2022) observed, reverse transcriptase PCR was employed owing to its high sensitivity, high specificity, efficiency and especially its ability for identifying the ND virus. Out of 44 hens investigated, Worku *et al.*, (2022) showed that 17 had the Newcastle disease virus in their lungs. By using reverse transcriptase real-time PCR, we were able to confirm the presence of viruses in 17 of the 18 samples. Exposure of the hens to Newcastle disease was established by amplification of the matrix gene in isolate samples. This result was consistent with the findings of Alsaahmi *et al.*, (2018), which isolated and identified the virus from samples suspected of having Newcastle disease using reverse transcriptase real-time PCR. All 17 isolates tested positive for NDV when their RNA was analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR), as reported by Ahmed & Odisho, (2018), positive for NDV by RT-PCR (80%). Primers designed to target the F gene were thought to be effective for spotting common NDV strains in this investigation. However, there may be other strains in the area, so this conclusion should be treated with caution. This seems to be particularly evident when examining the results of F gene testing, which showed an extremely low positive rate. Lastly, the study's non-random sample limits the generalizability of its findings but does not alter the connections found between infection and risk variables. Contrarily, we found that certain instances that looked like ND were not picked up by the F gene primers over the course of the investigation. It has been observed that mismatches are easily detected by approaches based on probe/primer hybridization to a particular location, leading to frequent false-negative findings. There have been substantial updates to the genomic data of NDV in the databases, and novel genotypes have been identified, which may not be detectable by the primer/probes used in the current work, which were disclosed in 2004 and 2009, respectively. The assay's F gene primer designers also noted that their oligos did not perfectly match the sequences of all APMV-1 isolates in the databases at the time (Wise *et al.*, 2004). 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.

CONCLUSION

It was concluded that, in order to effectively develop a control strategy against the illness, rapid test detection and identification of the viruses are essential. As a result, the RT-PCR molecular technique may be used for quick and confirmatory detection of NDV from any kind of ND epidemic. The RT-PCR assay also had a higher detection rate of NDV than the HI test. Since this is the case, introducing the molecular approach (RT-PCR) for quick and confirmatory detection of NDV from any kind of ND outbreak at the field level of Iraq is feasible.

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