

Alternative method using Real Time PCR for evaluation of inactivated Newcastle disease viral vaccine

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The present work aims to establish a new alternative protocol to evaluate the *in vitro* potency of inactivated Newcastle disease virus vaccine using Real Time PCR. Aqueous phases of seven inactivated Newcastle disease virus vaccines batches from different manufacturers were extracted by isopropyl myristate. The Newcastle disease virus antigen of each vaccine sample was determined by a standard Real Time PCR assay. Vaccines were inoculated into separate groups of 3-week-old specific pathogen free chickens using the recommended dose of vaccine. The immunogenicity of each vaccine was assessed by the Newcastle disease virus hemagglutination inhibition antibody titers. Individual serum samples were collected 4 weeks post vaccination, then vaccine efficacy and protection rates were recorded after challenge test of birds vaccinated with the virulent Newcastle disease virus. There is the possibility of using the Real Time PCR as an *in vitro* assay for vaccine evaluation. The Cycle Threshold values ranged between 21.17 and 25.23. The hemagglutination inhibition titers ranged between 7.1 log₂ to 6.2. The comparison between the Cycle Threshold values of the antigen extracts and the results of the challenge test and *in vivo* hemagglutination inhibition assays using sera from vaccinated birds showed a correspondence between the *in vitro* and *in vivo* results.

Keywords: *in vitro*; inactivated vaccines; Newcastle disease virus; Real Time PCR; vaccine potency.

Introduction

Newcastle disease (ND) is an infectious highly contagious disease of poultry. It is caused by virulent strains of Newcastle disease virus (NDV). In the past, it was known as avian paramyxovirus serotype 1 and characterized mainly by damaging to the central nervous system and digestive tract⁽¹⁾ with high mortality rates. According to the international reports, there were about four fatal outbreaks of ND around the world that caused huge harm to poultry industry and international trade.⁽²⁾ NDV is an ancient endemic avian disease in Egypt. The current policy for its control depends on vaccination.⁽²⁾

An *in vitro* potency method can be used to evaluate inactivated, oil-adjuvanted NDV vaccines. The vaccine batch can be judged as approved or rejected the amount of hemagglutinin-neuraminidase (HN) antigen per dose

in the aqueous phase of the vaccine, post extraction by isopropyl myristate (IPM).⁽³⁾

Hundreds of oil-adjuvanted monovalent and combined NDV vaccines are registered annually at the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) to be evaluated for their quality. Routine *in vivo* evaluation procedures are applied according to Egyptian Standards Regulations for Evaluation of Veterinary Biologics.⁽⁴⁾ The current standard *in vivo* method to evaluate NDV vaccines includes vaccination of chickens with the recommended dose and route of vaccine, collection of blood samples at 28th day post vaccination for serological hemagglutination inhibition (HI) testing and challenge test with ND virulent virus.

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In vivo methodology for potency testing is time-consuming and not cost-effective. Hence, the establishment and development of new *in vitro* potency assays for inactivated NDV vaccines is essential to improve the monitoring and approval process of vaccine before release to markets. This target is of high priority to the CLEVB to reduce the time required for vaccines evaluation. The present study illustrates the application of Real Time PCR (RT-PCR) to evaluate NDV vaccine efficacy, by determination of NDV antigen in inactivated vaccines.⁽⁵⁾ The RT-PCR assay is an ideal, simple and fast method with reduced risks of contaminations. Additionally, RT-PCR is a more specific and sensitive method.⁽⁶⁾

Materials and Methods

Vaccines

Seven batches of different inactivated NDV vaccines were randomly selected to vaccinate seven groups of 3-week-old chicks according to Egyptian Standards Regulations.^(4,7) The same batches were used for ND antigen recovery technique for further antigen determination. The vaccines used differed in the virus strains contained, inactivation method and composition.

Chickens

Two hundred, 3-week-old specific pathogen free (SPF) chickens were obtained from the SPF Egg Production Farm, Koum Osheim, El-Fayoum, Egypt.

Virus

To assure the potency of vaccines, chickens were challenged with virulent NDV type 7 NDV-B7-RLQP-CH-EG-12, accession No. KM288609. This virus strain was also used for hemagglutination (HA) and HI tests. It was kindly obtained from Strain Bank of CLEVB, Abbasia, Cairo, Egypt.

Potency test

A vaccine challenge test was conducted using 3-week-old SPF chicks that were divided into eight groups (25 each group) and kept in separate isolators. Chickens in groups 1 to 7 were vaccinated with ND vaccines; group 8 was the non-vaccinated control group. Chickens were vaccinated as recommended on the vaccine label and

kept isolated under observation for at least 28 days. On day 28th post vaccination, blood samples were collected for haemagglutination inhibition (HI), then, the vaccinates and at least 10 unvaccinated chickens that had been kept isolated as controls were challenged with Embryo infectious dose 50 (EID₅₀)/0.1 mL (per bird) of the virulent strain of NDV Genotype 7 (virulent NDV type 7 NDV-B7-RLQP-CH-EG-12) supplied by the CLEVB strain bank; the vaccinates were observed each day for 14 days. If at least 90 percent of the controls do not show typical signs of ND or die within 6 days, the test may be repeated. If at least 90 percent of the vaccinated do not remain normal, the vaccine is unsatisfactory as described in Office International des Epizootics (OIE).⁽⁷⁾ The protection percentage of the seven vaccines were measured after vaccination; the percentage of protection should be greater than or equal to 90%.

Hemagglutination inhibition test

Blood samples collected at 28th day post vaccination were used for serological tests to determine the NDV antibody titer for each vaccine by HI test as described in OIE.⁽⁷⁾ Two fold serial dilution of serum samples from 1/2 to 1/2048 were applied against 4 HA units of ND antigen 10⁶ EID₅₀/0.1 mL using HA test.⁽⁷⁾ The geometric mean HI antibody titer was calculated.^(8,9) The mean HI titer should be at least 6 log₂.

ND antigen recovery and extraction

Six mL of each oil-emulsion vaccine were mixed and shaken well with 24 mL of IPM for 2 min at 2500 rpm/min, then centrifuged for 10 min at 2500 rpm at 4°C. The resultant aqueous phase was collected separately for each vaccine for ND antigen determination by RT-PCR.⁽¹⁰⁾

RNA extraction

RNA extraction was done for seven extracted ND antigen using QIA amp viral RNA Mini kit (Qiagen, Germany, GmbH). One hundred and forty µL of the ND antigen extract were incubated with 5.6 µL of carrier RNA and 560 µL of AVL lysis buffer at room temperature for 10 min, then, 560 µL of absolute ethanol was added to the resultant lysate to be washed and centrifuged as recommended by the manufacturer's

Table 1. Oligonucleotides, primers and probes (Metabion, Germany).⁽¹²⁾

Gene	Primer/probe sequence 5'-3'
ND (M)	M+4100 AGTGATGTGCTCGGACCTTC-3'
	M-4220 CCTGAGGAGAGGCATTTGCTA-3'
	M+4169 (FAM)TTCTCTAGCAGTGGGACAGCCTGC(TAMRA)-3'

instruction. Nucleic acid was then eluted by 60 μ L of AE elution buffer.

RT-PCR amplification

The reaction was done in MX3005P RT-PCR machine in a final volume of 25 μ L containing 3 μ L of RNA template, 12.5 μ L of 2x QuantiTect Probe RT-PCR Master Mix, 8.625 μ L PCR grade water, 0.25 μ L of each primer (50 pmol conc.) and 0.125 μ L of each probe (30 pmol conc.) and 0.25 μ L of QuantiTect RT Mix (Table 1). The Reverse transcription was applied at 50°C for 30 min. Afterwards, primary denaturation was adjusted at 94°C for 15 min, 40 cycles of denaturation at 94°C for 15 sec were done to be followed by annealing at 55°C for 30 sec and finally, extension at 72°C for 10 sec.

All reactions that recorded Ct values were considered positive and reactions that did not record Ct values were considered negative.⁽¹¹⁾

Ethical approval

All methods used in the study were performed according to relevant guidelines and regulations. All experiments were carried according to ARRIVE 2.0 guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) in the Faculty of Veterinary Medicine, Cairo University under the code (VetCU01102020217).

Results

Hemagglutination inhibition test and efficacy results (*in vivo* potency)

Seven batches of different inactivated NDV vaccines were evaluated at CLEVB for their potency by traditional methods (vaccination, serological and challenge test) and satisfactory results were obtained (Table 2). These data were considered for further assessment of an alternative method.

To assure the potency of each vaccine, chickens were challenged with the virulent NDV Egyptian isolate of genotype VII. All tested batches protected vaccinated SPF chickens, the percentage of protection ranged from 90% to 100% (Table 2).

The protection percentage shows correspondence with HI antibody titers of sera from vaccinated chickens; the vaccine batch (A) recorded the highest titer 7.1 log₂ with 100% protection, batches (B and C) 6.5 log₂ with 95 % protection, batches (D, E and F) 6.3 log₂ with 90% protection and batch (G) recorded 6.2 log₂ with 90% protection. These results are considered the starting point of the study to find a coincidence between immune response of each vaccine and the antigen determination (by RT-PCR) as an alternative method to the traditional potency assay.

Table 2. Humoral immune response and protection rates of SPF chickens vaccinated with inactivated NDV vaccines.

Test	Vaccine lab symbol							Control
	A	B	C	D	E	F	G	
HI titer (log ₂)	7.1	6.5	6.5	6.3	6.3	6.3	6.2	-
Protection %	100	95	95	90	90	90	90	10

Protective HI antibody titer ≥ 6 (log₂). Protection level (%) of challenge test $\geq 90\%$.

Table 3. Mean Ct values of the *in vitro* Real Time PCR for extracted ND antigen.

Sample No.	HI Titer	Result	Ct value
A	7.1	+	21.17
B	6.5	+	22.75
C	6.5	+	22.84
D	6.3	+	23.24
E	6.3	+	24.09
F	6.3	+	24.81
G	6.2	+	25.23
Control Group	-	-	No Ct

Ct values > 40 reveals negative results.

Real Time PCR

NDV antigens of the tested vaccines were efficiently extracted by oil adjuvant breakdown using IPM to be identified and determined by RT-PCR. Different antigen titers were found (Table 3), but in general, the higher antigen amounts the higher serological response.

When RT-PCR was used to determine NDV antigens it was recorded for vaccine batches G, F, E, D, C, B 25.23 Ct, 24.81 Ct, 24.09 Ct, 23.24 Ct, 22.84 Ct, 22.75 Ct, respectively and for batch A, 21.17 Ct (Table 3). It is noticed that there was coincidence between the HI titers and Ct values.

Coincidence between *in vivo* and *in vitro* alternative methods

The coincidence between the *in vivo* and *in vitro* evaluation methods obtained for different vaccines batches was outlined in Figure 1.

Discussion

The establishment of alternative *in vitro* potency methods for batch release of inactivated NDV vaccines is a high priority for the Egyptian CLEVB to reduce the cost and save time in relation to *in vivo* potency testing. Strict batch-to-batch potency assays of all NDV vaccines are applied by CLEVB. Evaluation of

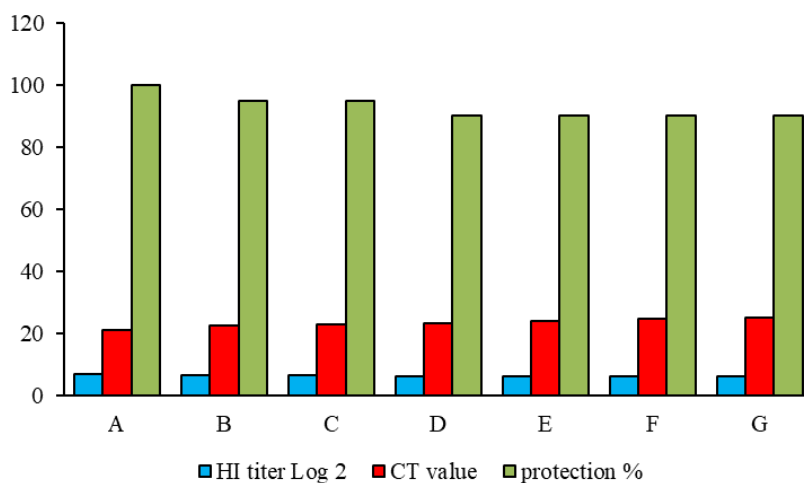


Fig 1. Relation between *in vivo* HI and challenge test and *in vitro* alternative RT-PCR methods.

inactivated avian viral vaccines, traditionally based on vaccination of 3-week-old SPF chickens with a full dose, is routinely performed to assess the potency of commercial vaccines. When *in vivo* potency tests were applied, HI antibody titers $\geq 6 \log_2$ were obtained in serum samples collected 4 weeks post vaccination and at least 90% protection, indicating a satisfactory protection rate. The vaccinated chickens were subjected to challenge test with further observation for 6-day, after these 6 days the trial was considered valid.

According to previous data, the evaluation procedure needs at least 34 days, which is a long period, therefore it is necessary to minimize the evaluation time.^(5,7)

The known cross-reaction between some avian paramyxoviruses and NDV leads to uncertainty in ND serological assays, like the HI test. Conventional serological tests have an insurmountable barrier for typing. From this point, there is a need to establish a standard molecular assay to accurately detect and quantify NDV antigen. RT-PCR assays play an important role in rapid identification of NDV and also allow its accurate distinguishing from other closely related avian paramyxovirus pathogens. In this study, RT-PCR was introduced as an alternative accurate time-saving protocol.^(12,13)

The study discusses the use of RT-PCR as an *in vitro* alternative method for evaluation of inactivated NDV vaccines for poultry. RT-PCR was applied for NDV antigen determination parallel with traditional immunogenic and potency tests to find a coincidence between the *in vivo* and *in vitro* techniques. The inactivated NDV antigens could be isolated from water-in-oil emulsion inactivated vaccines by IPM and quantified by RT-PCR. Antigen isolation facilitated the comparison between the amounts of NDV antigen and the titer of ND antibody response post vaccination with seven different inactivated ND vaccines from different manufacturers.

From the mentioned results, amount of NDV antigen looked to be a considerable indicator of serological immune response, as it was clear that the high antigen content resulted in a high antibody titer. These results are in agreement with a previous study⁽¹⁴⁾ that demonstrated the potential of the RT PCR assay as an

alternative rapid method to traditional virus titration for evaluation of inactivated viral vaccines. This comparison may be an additional evidence of the *in vivo* potency and efficacy of the inactivated NDV vaccine. The study considered the results of seven batches of inactivated NDV vaccines which were previously evaluated at CLEVB for their efficacy and potency by using traditional methods including HI and challenge test. The seven vaccine batches revealed satisfactory results for the protection percentage (equal to or greater than 90%) and protective HI antibody titer (greater than $6 \log_2$).⁽¹⁵⁾

Additionally, serological and vaccination challenge tests are known to have shortcomings because the immune response and protection are measured at specific time point post vaccination under specific standard conditions. On the other hand, the *in vitro* antigen determination assay is a suitable method for this purpose, being simple, relatively cheap and robust and help avoiding the animals use.⁽³⁾

Globally, there is few antigen determination assays that are accepted as alternatives to potency tests for inactivated vaccines. In Europe, antigen determination is not accepted as an alternative to the *in vivo* potency assay of veterinary vaccines,⁽¹⁶⁾ but in USA, these assays are used instead of traditional assays for inactivated viral veterinary vaccines.⁽¹⁷⁾

The study recommends the application of Real Time PCR as a reliable technique for evaluation of inactivated ND vaccine efficacy. Furthermore, the relationship between the *in vitro* amount of antigen and protection percentage has to be calculated for each newly registered vaccine, as it is important to make general criteria for the antigen determination assays by RT-PCR and HI antibody titer for each vaccine.⁽³⁾

Several previous studies have been conducted to apply *in vitro* potency assays to replace the time consuming *in vivo* assays, by determination of the NDV antigen content by RT-PCR of tested vaccines after antigen extraction by IPM.^(18,19)

The present study was established to perform an easy, accurate and fast substitutive protocol to the conventional one. Seven inactivated NDV oil-

adjuvanted vaccine samples of batches manufactured by different companies were treated with IPM, resulted in successful extraction of the NDV antigen which was determined using RT-PCR assay.

RT-PCR successfully detected NDV antigen in all extracted vaccine samples which showed a correspondence between the antigen amount measured by RT-PCR assay and the HI titer of collected serum samples at 28 days post vaccination. Hence, the *in vivo* challenge potency test was applied to confirm the effectiveness of the seven selected batches which recorded Ct values between 21.17 and 25.23 to be used for immunogenicity assays. The obtained results are in agreement with those obtained by a previous study.⁽¹⁰⁾

Previous studies reported a correlation between the *in vivo* and *in vitro* potencies for different vaccines regardless the inactivation method. The vaccination challenge study did not reveal clear differences between the *in vivo* tests for the evaluated vaccines. But the vaccination serology assay revealed that the *in vivo* potency of the tested vaccines was different as the antibody titer ranged from 7.1 log₂ to 6.2 log₂. Additionally, the potency of the vaccines cannot exceed its limit, including a higher than adequate concentration of antigen administered.⁽³⁾

The RT-PCR technique is characterized by its ability to monitor exactly when the DNA amplification occurs. Using a standard curve allows the analysis of the unknown templates. When comparing conventional methods to RT-PCR, several advantages are found as a high sensitivity, rapidity, low false positives and the accurate analysis. Moreover, RT-PCR is a powerful technique for safe determination of gene expression in addition to its specificity, sensitivity and reproducibility without need to animal immunization.^(12,13)

On the vaccine evaluation scale, the HI test cannot differentiate vaccinated from infected chickens which to some extent may interfere with the judgment reports by releasing the vaccine batch as satisfactory, but in fact the chicken might be infected.

Conclusions

RT-PCR can be considered a good, fast, safe, accurate, low cost and time saving tool and has the ability to

replace the traditional efficacy and potency methodology according to the results obtained. Antigen determination by RT-PCR could replace the *in vivo* potency method for evaluation of NDV inactivated vaccines.

Conflict de interest

The authors declare that there is no conflict of interest.

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Author's contributions

Mounir El Safty: designed the experiments.

Hala Mahmoud: performed the experiments and wrote the manuscript.

Reem A Soliman: performed the experiments and wrote the manuscript.

All authors reviewed and approved the final version of this manuscript for publication.

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Método alternativo de PCR en tiempo real para la evaluación de la vacuna vírica inactivada contra la enfermedad de Newcastle

Resumen

El presente trabajo pretende establecer un nuevo protocolo alternativo para la evaluación *in vitro* de la potencia de la vacuna de virus inactivado contra la enfermedad de Newcastle mediante PCR en tiempo real. Las fases acuosas de siete lotes de vacunas inactivadas contra el virus de la enfermedad de Newcastle de distintos fabricantes se extrajeron mediante miristato de isopropilo. El antígeno del virus de la enfermedad de Newcastle de cada muestra de vacuna se determinó mediante un ensayo estándar de PCR en tiempo real. Las vacunas se inocularon en grupos separados de pollos libres de patógenos específicos de 3 semanas de edad utilizando la dosis recomendada de vacuna. La inmunogenicidad se evaluó para cada vacuna mediante los títulos de anticuerpos de inhibición de la hemaglutinación del virus de la enfermedad de Newcastle. Se recogieron muestras individuales de suero 4 semanas después de la vacunación y, a continuación, se registraron la eficacia de la vacuna y los índices de protección tras la prueba de reto de las aves vacunadas con el virus virulento de la enfermedad de Newcastle. Existe la posibilidad de utilizar la PCR en tiempo real como ensayo *in vitro* para la evaluación de vacunas. Los valores del umbral de ciclo oscilaron entre 21,17 y 25,23. Por otra parte, los títulos de anticuerpos inhibidores de la hemaglutinación oscilaron entre 7,1 \log_2 y 6,2. La comparación entre los valores del umbral de ciclo de los extractos de antígeno con los resultados correspondientes de la prueba de reto y los ensayos de inhibición de la hemaglutinación *in vivo*, utilizando sueros de aves vacunadas, demostró una fuerte correspondencia entre los resultados *in vitro* e *in vivo*.

Palabras clave: *in vitro*; vacunas de productos inactivados; virus de la enfermedad de Newcastle; Reacción en Cadena en Tiempo Real de la Polimerasa; potencia de la vacuna.

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