Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Determination of efficacy in inactive Newcastle disease vaccines with an *in-vitro* method as an alternative to *in-vivo* methods

In-vivo yöntemlere alternatif olarak *in-vitro* yöntemle inaktif Newcastle hastalığı aşılarında etkinlik tayini

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ABSTRACT

Objective: In this study, applicability of an *in-vitro* ELISA method, which reduces or eliminates the need for animal trials in establishing the efficacy of inactive Newcastle disease vaccines was investigated.

Methods: For this purpose, the efficacy of nine (9) inactivated Newcastle disease vaccines and two non-Newcastle related vaccines (negative controls) were evaluated by *in-vivo* and *in-vitro* methods. As *in-vivo* tests, vaccines were applied to the Spesific Pathogen Free (SPF) chicks and antibody response was determined via hemagglutination inhibiton test. As *in-vitro*, antigen amount in vaccines were calculated with a sandwich ELISA test based on the determination of hemagglutinin and neuraminidase levels developed for inactive Newcastle vaccines. Relative potency values of vaccines were calculated by using the parallel line analysis method with Combistats statistical analysis software program by using optical density results obtained from ELISA test.

Results: Hemagglutination inhibition test results were found to be between $2^{3,3}$ and $2^{6,1}$; immunization/

ÖZET

Amaç: Bu çalışmada, inaktif, Newcastle hastalığı aşılarının etkinliğini belirlemede hayvan denemelerine olan ihtiyacı azaltan veya ortadan kaldıran *in-vitro* bir ELISA yönteminin uygulanabilirliği araştırılmıştır.

Yöntem: Bu amaçla, dokuz (9) inaktif Newcastle hastalığı aşısının ve iki Newcastle ile ilişkili olmayan aşının (negatif kontroller) etkinliği *in vivo* ve *in-vitro* yöntemlerle değerlendirilmiştir. *In vivo* testler olarak Spesifik Patojensiz (SPF) civcivlere aşılar uygulanmıştır ve hemaglütinasyon inhibisyon testi ile antikor yanıtı belirlenmiştir. *İn vitro* olarak, aşılardaki antijen miktarı, inaktif Newcastle aşıları için geliştirilen hemaglutinin ve nöraminidaz seviyelerinin belirlenmesine dayanan bir sandviç ELISA testi ile hesaplanmıştır. Aşıların nisbi potens değerleri, ELISA testinden elde edilen optik yoğunluk sonuçları kullanılarak Combistats istatistiksel analiz yazılımı programı ile paralel çizgi analiz yöntemi kullanılarak hesaplanmıştır.

Bulgular: Hemaglütinasyon inhibisyon testi sonuçları $2^{3,3}$ ile $2^{6,1}$ arasında bulunmuş; bağışıklama / meydan

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Geliş Tarihi / Received : 17.08.2021
Kabul Tarihi / Accepted : 28.03.2022

DOI ID : 10.5505/TurkHijyen.2023.84704

Kars M, Tekintaş Y, Çöven F, Dakman A, Öztürk İ, İçin S, Hoşgör Limoncu M. Determination of efficacy in inactive Newcastle disease vaccines with an *in-vitro* method as an alternative to *in-vivo* methods. Turk Hij Den Biyol Derg, 2022; 80(1): 33 - 42

challenge results ranged between 56 and 136 PD_{50} and ELISA test results were between 6,02 and 98,23 antigen units. A correlation was found between the ELISA test and *in-vivo* methods. Sensitivity and specificity were found to be %89, %100 respectively.

Conclusion: In our study according to these results, we have demonstrated the ability of ELISA method to give similar results on the same samples, its compatibility with the standard procedure and its applicability under laboratory conditions.

Key Words: *In-vivo, in-vitro,* Newcastle disease, relative potency, vaccine

okuma sonuçları 56 ve 136 PD50 arasında değişirken, ELISA test sonuçları 6,02 ve 98,23 antijen birimleri arasında olduğu görülmüştür. ELISA testi ile *in vivo* yöntemler arasında bir korelasyon belirlenmiştir. Duyarlılık ve özgüllük sırasıyla %89 ve %100 olarak bulunmuştur.

Sonuç: Bu sonuçlara göre, laboratuvar şartlarında ELISA metodunun uygulanabilirliği, standart prosedüre olan uyumu ve aynı örnekler üzerinde benzer sonuçlar verebilme yeteneği gösterilmiştir.

Anahtar Kelimeler: *İn-vivo, in-vitro*, Newcastle hastalığı, relatif potens, aşı

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease that can be seen in domestic and wild bird species (1). Although there are clinically asymptomatic cases, the disease usually presents with symptoms of the digestive, respiratory and nervous system and rapidly progresses to a fatal stage (5). It is one of the most important poultry diseases in the world, with its high morbidity and mortality, which rates up to 100% (5). This enveloped, single-stranded RNA virus is endemic in many parts of the world and also can cause outbreaks in domestic poultries. There is no effective antiviral for the treatment of Newcastle disease (20). Therefore biosecurity and hygiene measures and vaccination practices should be carried out in harmony since the disease can infect different domestic and wild birds which also can asymptomatically carry the virus and contribute to the spreading (4).

Every year, hundreds of batches of inactivated ND monovalent or combined vaccines are used. Unlike pharmaceuticals, each batch is considered unique as they are derived from living organisms. Manufacturers and competent authorities should check each batch to ensure product consistency, safety, and efficacy (15). One of the most important features that determine the test method is the type of vaccine. In general, *in-vitro* titration tests and colony counting determine the efficacy of live vaccines. However, in inactive vaccines' efficacy is determined by *in-vivo* methods such as immunization-challenge, toxin neutralization, and immunization-serology tests (18). However, ethical concerns about animal welfare, it is encouraged to reduce the number of animals used in, or use alternative *in-vitro* methods (11, 13, 18, 22).

In this study, applicability of an in-vitro ELISA method, which reduces or eliminates the need for animal trials in establishing the efficacy of inactive Newcastle Disease Vaccines was investigated.

MATERIAL and METHOD

Vaccines

In the study, nine oil-emulsion inactivated vaccines from different companies containing different lentogenic ND virus strains. Oil-emulsion inactivated vaccines of reovirus and *Avibacterium paragallinarum* were included to detect possible false reactions. The efficiency of the vaccines was evaluated by *in-vivo* immunization-serology, immunization-challenge test and in-vitro antigen ELISA. The contents of the vaccines used are shown in Table 1.

Table 1. Vaccines and their ingredients

Vaccine No:	Vaccine strain	Vaccine type	Inactivation agent
#1	Lasota strain	Combined	Formaldehyde
#2	VH strain	Monovalent	Formaldehyde
#3	Clone 30 strain	Combined	Formaldehyde
#4	Lasota strain	Combined	Formaldehyde
#5	Lasota strain	Combined	Formaldehyde
#6	Ulster 2C	Monovalent	Formaldehyde
#7	Lasota strain	Combined	ß-propiolactane
#8	Lasota strain	Monovalent	Ethylene-imine
#9	Lasota strain	Combined	Formaldehyde
#10	Reovirus strain	Multivalent	Formaldehyde
#11	*Avibacterium paragallinarum* serotype A, B, C	Multivalent	Formaldehyde

Immunization-serology test

For each vaccine sample, 10 chicks that were 21-28 days old and obtained from the SPF flock were used. At least five chicks were left unvaccinated as a control group. One-fiftieth of the vaccine dose was given by intramuscular injection (IM) to 10 SPF chicks in each group with micro injectors. Seventeen-twenty-one days after vaccination, at least 1 ml of blood was taken from each chick and the sera were separated. Serum samples were kept at 56 °C for 30 minutes to inactivate the complement and stored at -20 °C. Antibody titers were calculated by hemagglutination (HA) and hemagglutination inhibition (HI) tests as specified in World Organization for Animal Health (OIE), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Section 3.3.14 (21). The effectiveness criterion of vaccines was determined as HI titers 4.0 log2 and above in the vaccinated group and 2.0 log2 and below in the unvaccinated group.

Immunization-challenge test

In the test, the protective dose (PD_{50}) of the vaccines was calculated based on the survival rates of the SPF chicks after immunization. The experiments were done with the recommendations of the European Pharmacopoeia monograph No. 0870 (21). Test groups include 20 chicks that were 21-28 days old and obtained from the SPF flock. The chicks were vaccinated intramuscularly at varying rates (1/12.5, 1/25, 1/50, 1/100, 1/200) of the vaccine dose. Ten chicks were left as the control group. After 17-21 days

of vaccination, all groups, including the control group, were intramuscularly epruvated with velogenic AA-1 (Herts 33/56) containing 6 log10 ELD50 / dose and placed in the isolator. Chicks that survived without showing Newcastle disease symptoms were identified in all groups for 14 days after the application and protective dose (PD_{50}) was calculated according to the Reed and Muench method (23). For the control of validity of the test, all animals in the control group must have died within six days after epruvation.

Extraction Process of Vaccines and ELISA

For including the vaccines in the ELISA test, the antigen must first be released to the water phase by the degradation of the emulsion. For this purpose, 8 ml of isopropyl myristate (IPM) and 2 ml of vaccine were mixed in a 15 ml tube. The suspension then vortexed for 1 minute. Following, the mixture was centrifuged for 10 minutes at 2500 rpm at 8 °C by using a cooled centrifuge. Then the liquid part (oil + IPM) remaining in the upper part of the tube was removed by pipetting (8). The double-antibody sandwich (DAS) ELISA was used to determine the potency of inactive ND vaccines, based on measuring the HN protein's amount of the AA-1 serotype. The relative potency of the vaccine samples was determined as the antigen unit (AU) by comparing the reference antigen (6AU/vial) with known HN protein content (7). In ELISA experiments, ND virus HN protein-specific MoAb antibody was used for coating, Horse radish peroxidaseǁ (HRPO) conjugated ND virus HN specific MoAb was used as the detection antibody. The results of the ELISA experiments were analyzed with the current version of the Combistats Statistical Analysis Software Program provided by EDQM. The OD values of the samples and the reference antigen were converted into logarithmic values using the parallel line analysis method, the relative potency (AU/ml).

Comparison of the methods

The relationship between immunization-serology, immunization-challenge, and ELISA results was evaluated using Minitab Statistical Program. The coefficient of variation (% CV) between OD values of samples and % CV for the relative potency values obtained from six replicates of six samples on different days was calculated. The sensitivity, specificity, and relative accuracy rate of the ELISA were calculated based on *in-vivo* test results (10).

RESULTS

Hemagglutination Inhibition Test Findings

According to the HI test results of chicks used in the control of ND vaccines. Five of nine ND vaccines produced 2^4 or fewer antibody responses, and four produced antibody responses above this value. Two vaccines that do not contain ND virus components did not produce any response as in the control chicks. Results were summarized in Table 2.

Immunization-Challenge Test Findings

When the vaccine series that revealed an antibody response at an average level of $2^{3,3}$, $2^{3,4}$, $2^{3,3}$, $2^{3,5}$, and $2^{3,9}$ (below 2^4) according to the results of HI test performed for each vaccine were evaluated for the chick number that survived after the immunization-challenge test, they exhibited 56, 60, 70, 75, and 71 PD_{50} protection level, respectively. When the remaining four vaccine samples were taken into the HI test, an average of $2^{4,1}$, $2^{4,8}$, $2^{4,4}$, and $2^{6,1}$ antibody responses were obtained, and in these vaccine series, protection at the level of 100, 108, 100, and 136 PD_{50} values was determined, respectively. A significant correlation was observed when HI levels and PD_{50} values were compared with each other (r^2 = 0.95) and the results were found statistically significant ($p<0.001$). All unvaccinated chicks died by the 4th day of the experiments (Table 2).

ELISA Findings

The relative potency values of vaccines containing ND components, found between 6.02 and 98.23 AU/dose. The relative potency of the two vaccines, which were the negative control, was found as 0.19 and 0.34 AU/dose. The correlation (r^2 = 0.93) was

found between the ELISA test and the Immunization-challenge test ($p < 0.001$). In addition, when all data were analyzed, a significant relationship was found between the ELISA and *in vivo* methods with the %89 sensitivity and %100 spesificity. The ELISA test results of all vaccines studied are given in Table 2.

Table 2. Test results of vaccines

Vaccine No:	HI test results		PD_{50}/dose results		ELISA
	Vaccinated	Control	Result	Acceptance Limit	
#1	$2^{3,3}$	0	56	50	6,02
#2	$2^{3,4}$	0	60	50	8,93
#3	$2^{3,3}$	0	70	50	8,55
#4	$2^{3,5}$	0	75	50	10,33
#5	$2^{3,9}$	0	71	50	12,01
#6	$2^{4,1}$	0	100	50	36,91
#7	$2^{4,8}$	0	108	50	75,67
#8	$2^{4,4}$	0	100	50	36,75
#9	$2^{6,1}$	0	136	50	98,23
#10	0	0	-	-	0,19
#11	0	0	-	-	0,34

DISCUSSION

Although challenge practices directed at determining potency in inactive ND vaccines clearly demonstrate the effects of all vaccine components (adjuvants, emulsifying agents) on the immune system, the time from the initiation to the termination of the test takes approximately two months, a large number of (min.70) SPF chicks are used and evaluation of test results is based on observation of disease symptoms and survival levels in animals. The alternative ELISA test is based on calculating the relative potency by directly comparing the amount of antigen in inactive ND vaccines with a reference antigen without any immunization. In our study, the compatibility between *in-vivo* and *in-vitro* test methods used to evaluate

the effectiveness of vaccines was investigated. Five of the nine ND vaccines which were subjected to the *in-vivo* immunization-serology protocol, gave a serologic response which was 2^4 or below. When these afore-mentioned vaccines were tested through immunization-challenge; they were all found have a value above 50 PD50. There are a lot of studies in which serologic response to vaccination is similarly evaluated, it is significant that the serological response level in which protection does not occur is below 2^2. it was found out that none of the vaccines gave a response below 2^3, only a certain group of vaccines gave results below 2^4 which is the limit set by the European Pharmacopoeia, and even these vaccines were found to provide protection over 50 PD50. In the monograph 0870 of European Pharmacopoeia, it is required to conduct immunization-challenge to decide the efficacy of the inactive ND vaccine when serological tests do not yield the necessary antibody titers (21). The vaccines with HI titers between 2^3 and 2^4 obtained in our study met the Pharmacopoeia acceptance criteria of 50 PD50, it was thought that values below 2^4 could be due to the difficulties in vaccine administration. As administration doses contained very low vaccine doses such as a 1/50 ratio, vaccine delivery was possibly curtailed. In our study, a significant correlation was observed when HI levels and PD50 values were compared with each other (r^2 = 0.95) and the results were found to be statistically significant ($p < 0.001$). There are studies with similar results to our results in *in-vivo* tests performed to determine the effectiveness of ND vaccines (2, 14, 24).

The double antibody sandwich ELISA test, which constitutes the in-vitro part of our study, is based on the HN antigen in the vaccine conjugating with the specific monoclonal antibody and the measurement of the relative potency after the antigen antibody complex is compared with a reference sample with known antigen amount, without the need for immunization and challenge in SPF chicks. It has been reported that similar vaccine series, products containing virus or bacterial subunits as well as microbial cultures can be used as reference products (17). RP values were calculated with parallel line analysis method using Combistats software 5.0 program using OD values obtained in the ELISA test of ND vaccines and they were between 6.02 AU / dose and 98.23AU / dose. In a previous report the minimum amount of antigen that vaccines should have in order to be able to use the ELISA method in potency testing of inactive ND vaccines was found to be 7 AU/dose (7). When this study was taken into consideration, all ND vaccines except one, had sufficient antigen amount. In our study, eight of nine ND vaccines were above 7 AU, and only one vaccine with a value of 6 AU remained below the limit even though it was very close and had sufficient PD50 results. In our study, a significant correlation (r^2 = 0.93) was found between the ELISA test and the immunization-challenge test ($p < 0.001$). There are studies in which the efficacy of inactivated Newcastle disease vaccines is investigated by comparing *in-vitro* and *in-vivo* tests (serological and challenge), and the results obtained from our study are similar to the results of the researchers (6, 19). It was thought that the relatively insufficient relative potency value of 6 AU in one vaccine may be due to the inactivation agent (formaldehyde) or due to the failure in separating the oil phase from the water phase during the extraction process, causing the oil phase to have a masking effect even though the antigen amount is sufficient. In a study on inactivation agent (formaldehyde), it was emphasized that in vaccines using formaldehyde, less antigen was detected compared to ß propiolactone and this was due to the mechanism of action of the inactivation agent (16). In another study, it was reported that insufficient AU amounts could be detected due to the inability to completely separate the oil phase from the water phase during the extraction process or the masking with the oil phase even though the amount of antigen was sufficient (3).

In our study, when we included a bacterial and a viral vaccine without the ND component into the ELISA test, RP was found at a much lower level (0.19

AU / dose and 0.34 AU / dose) than the detection limit of 7AU / dose, and with these results, the specificity of the test was verified. Maas et al. (19) reported that six different vaccines could be classified into insufficient, low and high antigen containing groups. PD50 results of vaccines with high antigen content were at maximum level, and the detection of different ND strain vaccines by the same MoAb was the most important outcome. In our study, vaccines containing different strains such as Lasota, Ulster and Clone30 were evaluated, and it was observed that all vaccine series produced sufficient RP response except for one vaccine sample, and vaccines with high RP had high PD50 and HI results. In the validation study carried out for ND vaccines, It was stated that the threshold antigen level to be used in determining the potency of vaccine series was calculated to be 7 AU / dose, this antigen level did not cause any false positive results in *in-vitro* measurements performed by the laboratories, and there were only a limited number of false negative results. With this validation study, the researchers revealed that vaccines with high PD50 also have high antigen unit (AU) values and they reported that this alternative method for antigen quantification can be included in the European Pharmacopoeia (7). In our study, while a limited number of false negative results (only in one sample) were obtained as a result of the ELISA test, there were no false positive results, and the specificity, sensitivity and relative accuracy rates were found to be high. In accordance with the validation study, it has been revealed that vaccines with high protective dose (PD50) have high antigen unit (AU) values.

It was suggested by EDQM that the control antigen which is the biological reference, should be determined by each laboratory that will use the ELISA test, and that the values obtained through repetitive studies should not be greater than the value determined during the method validation by 70% to 140%. In our study, the mean value of the control antigen was determined to be 3.2 AU /vial, and the alarm limit was 4.48 AU and 2.24 AU. When 18 results for the control antigen were evaluated, the lowest and highest values were 2.31 AU and 4.13 AU, respectively and therefore they were not out of the alarm limits.

For the validation of ELISA test method in our laboratory; six vaccines chosen from 11 different vaccine samples were studied repeatedly at different times along with the reference antigen. It was observed that the intra-analysis variation evaluated in the reproducibility study was less than 10%, excluding the variation coefficients of 13.87% and 14.25% seen in two vaccine samples. It was thought that plate wells going dry, pipetting errors and splashing between the wells could play a role in the intra-assay variation taking values above 10%. The coefficients of variation between the relative potency values of the vaccine samples that were analyzed on different days to form the reproducibility data were found to be less than 15% in all studies. These results seem to be compatible with the criterion that within-assay variation should not exceed 10% and inter-assay variation should not exceed 15% in ELISA studies (9).

In our study according to these results, we have demonstrated the ability of ELISA method to give similar results on the same samples, its compatibility with the standard procedure and its applicability under laboratory conditions. As a result, reliable test results may be obtained in a short time with the alternative ELISA method, which can be applied within the framework of the 3R approach without requiring the use of animals.

ACKNOWLEDGEMENTS

This study was produced from the Ph.D. thesis conducted in the Pharmaceutical Microbiology Program of Ege University Institute of Health Sciences (Acceptance date: 12.06.2019).

FINANCIAL SUPPORT

This study was supported by Ege University Scientific Research Projects Commission with project number 16/ECZ/008. General Directorate of Agricultural Research and Policies (TAGEM) also supported this project with the number TAGEM/HSGYAD/A/18/A5/P2/715.

ETHICS COMMITTEE APPROVAL

* The study was approved by the İzmir/Bornova Veterinary Control Institute Ethics Committee (Date: 26.04.2017 and Number: 71705440-170-2605).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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