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The Effect of Route of Administration and Dose on the Immunogenicity and Protective Efficacy of Newcastle Disease Thermostable Vaccine I_2 Strain

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Abstract

Newcastle disease (ND) is one of the most important diseases in poultry worldwide. It is highly contagious affecting chickens other avian species and wild birds causing higher economic losses. vaccination is the best control strategy for the disease and many commercial live and killed vaccines being commonly used in poultry farms. In the present study, the effect of route of administration and dose on the immunogenicity and protective efficacy of the thermostable Newcastle disease vaccine (I_2) was determined under Sudan field conditions. Three experiments were conducted to determine, firstly, the effect of routs of adminstration (namely intraocular (I/O), intranasal (I/N) and drinking water (D/W) and secondly, the dose of the vaccine (recommended dose and half, two and four times of the recommended dose) on the immunity of two weeks old commercial layer chicks under lab condition. In the third experiment, the immunogenicity of the vaccines I_2 "I/O, I/N and D/W" and Komarov "I/N and D/W" in three weeks old commercial layers and indigenous village chickens was determined under field condition. The results of the three experimental trials indicated that, protection against the challenge infection was attained when chicks received two doses of the I_2 vaccine via an intraocular route. The I/O route was found to be superior to the I/N which was confirmed to be better than the D/W route; as observed from the mean haemagglutination inhibition antibody (HI-Ab) titres and percentage of protection produced by the vaccine through these routes. When comparing the dose of the I_2 vaccine, it was proved that even half of the recommended dose resulted in good immune response and protection. Moreover, doubling or even four times of the recommended dose gave 100% protection without any adverse post vaccination reactions. Results of the field trials showed better Ab response of I_2 vaccine when given via an I/O route in three weeks old commercial layers and indigenous village chickens compared to K- vaccine of ND. Generally, at least HI antibody titre of 5.5 $\log_2$ is required for 100% protection against challenge infection.

Keywords

NDV, Thermostable Vaccine, Route, Dose, Safety, HI Test, Domestic Fowl, Sudan

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1. Introduction

Newcastle disease (ND) is a major health problem in poultry industry with a worldwide distribution (OIE 2012). Newcastle disease can be controlled through the administration of effective vaccines. Almost all the commercially available ND vaccines require refrigeration and begin to deteriorate rapidly after 1-2 hours if left at room temperature (around 25°C). Subsequently, because maintaining an adequate supply of refrigerated facilities may be a difficult task in many countries with unreliable electrical supplies, the development and large scale production of an

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effective thermostable ND vaccine seems imperative to support the poultry industry (Mahmood *et.al* 2014).

The modes of the disease transmission are correlated with the routes of the vaccine application. Transmission of the ND virus between birds is by either inhalation or ingestion (Alexander, 2009). It is clear that infective virus may be present in aerosols and that birds placed in an atmosphere containing such aerosols become infected (Alexander, 1991). This is the basis for application of live vaccines by spray and aerosol generators (Meulemans, 1988). Ingestion of contaminated faeces is also proved as the main method for bird to bird spread of virulent enteric ND virus. Similarly, this assumed the application of the vaccine via the drinking water and food (Alexander *et al.,* 1984).

The most common routes of application of ND live lentogenic vaccines worldwide are, via drinking water (D/W), intra nasal (I/N), intra ocular (I/O) and beak dipping (B/D). Application of live vaccines by sprays and aerosols is popular due to the ease with which large numbers of birds can be vaccinated in a short time (Alexander, 19991). In aerosol application, it is important to achieve the correct size of particles by controlling the conditions under which aerosol is generated (Allan *et al.,* 1978; Meulemans, 1988). Spraying of day old chicks may result in the establishment of infection in the flock with the vaccinal virus despite maternally derived immunity (Meulemans, 1988). Other routes of vaccine application such as wing-web (W/W) and intramuscular (I/M) injection are exclusively adopted for the mesogenic strain vaccines as they tend to display greater virulence for birds. Examples of these are Mukteswar, Komarov and Roakin. Mesogenic vaccines (Alexander, 1991).

The thermostable vaccine of ND- I_2 and V4 (Australian strains) are used to control ND in village chickens in Africa and Asia (Adwar and *Lukešová, 2008*). According to Spradbrow (1992), the best way of controlling ND virus in small family flocks in Asian countries was to administer the thermostable live vaccine strains orally. However, in some African countries these strains offered the best and most reliable protection at population level by eye drop method (Alders and Fringe, 1988; Anita, 1999).

The present study was designed to study the effects of route of application and dose of ND virus thermostable vaccine (I_2) experimentally under lab and field conditions of Sudan.

2. Materials and Methods

2.1. ND Viruses and Antiserum

A thermostable ND virus (I_2 strain) vaccine (Bensink and Spradbrow, 1999) kindly supplied by Prof Spradbrow P.B., University of Queenland, Australia. Was used for preparation of the vaccine.

A freeze-dried ND (LaSota strain) vaccine (Merial, France) was used for preparation of the HA antigen.

For Challenge, ND virus "chicken/Sudan/Obied/87" (genotype 3b- gene bank reference is GQ258674) previsouly isolated from an adult layer flock (Wegdan, *et.al* 2010) was used.

Reference ND antiserum "HA" (Deventer- The Netherlands) was used in HI test.

2.2. Chickens and Embryonated Eggs

Ninety- one-day-old chicks of Bovans breed and 9- day old embryonating chicken eggs were obtained from Coral Poultry Farm, Khartoum- Sudan. Eggs were used for vaccines preparation, while chicks were divided into nine groups and used to study the effect of routes of administration and dose on the immunogenicity of the thermostable Newcastle disease vaccine (I_2) under lab condition.

2.3. Vaccination Trials

The field vaccination trial was done within the framework of the project: *Improving Family Poultry Production in Africa* funded by IAEA/FAO. Adult indigenous chickens in five villages located north of Khartoum, Sudan and six (3- weeks old) commercial layers flocks were vaccinated with ND vaccines (I_2 and Komarov-strains) .

2.4. Preparation of the Master and Working Seeds of I_2 Vaccine

One lypholized ampoule of ND vaccine I_2 seed strain was reconstituted into 20 ml of PBS, then, distributed into vials as 1ml aliquotes, and stored at –20°C as master seed. For preparation of working seed, two vials of master seed were mixed together, then ten fold serial dilutions were prepared in PBS containing antibiotics mixture(penicillin, streptomycin, gentamycin and mycostatin), dilution 10^{-3} was used to inoculate forty 10 day old embryonating chicken eggs into allantoic cavity. Each egg received 0.1 ml of the inoculum, inoculated eggs were incubated at 37°C for 4 days. Embryos died 24 hours post inoculation were discarded, after 4 days, live eggs were chilled at 4°C for at least 2 hours. Then, allantoic fluid was harvested and the presence of the virus was detected by HA test and confermed as ND virus by HI test against reference ND virus antiserum. Quality control tests such as sterility' freedom of bacteria, mycoplasma, fungi and external viruses', safety and virus titration were done on the harvested allantoic fluid (OIE, 2012). Then, allantoic fluid was distributed into vials as 1ml aliquotes and stored at –20°C as working seed.

2.5. Preparation of I_2 Vaccine

The vaccine was produced in accordance with approval of the OIE Manual (OIE, 2012). 10 day-old embryonated chicken eggs were inoculated with the working seed at a dose of 0.1 ml per egg, using the same procedure for the working seed preparation, then, allantoic fluid was harvested from live embryos 4 days post inoculation and distributed into vials as 1ml aliquotes. Quality control tests such as sterility "freedom of bacteria, mycoplasma, fungi and external viruses", identity, safety,potency and efficacy were done according to OIE (2012). Allantoic fluid passes the QC. tests was used as ND vaccine.

2.6. Experimental Design

2.6.1. Experiment I

In this experiment, the effect of route of administration on the immunity to ND-I_2 vaccine was determined on 2-weeks old commercial layer flocks. Forty chicks were divided into 4 groups (G1, G2, G3 and G4) of ten chicks each. Chicks in group G1, G2 and G3 received two doses of ND-I_2 vaccine(at 2 and 4 weeks old) by I/N, I/O and D/W routes respectively. Group 4 was left as unvaccinated contact control. Four weeks post the 2nd vacccination, all chicks (vaccinated and unvaccinated) were bled from jugular vein using disposable syringe. The blood was left over-night at room temperature, then, a clear serum was separated by centrifuged at 1000 r.p.m for 10 minutes, stored at -20°C till used for ND virus antibody detection by means of HI test. Then the 4-groups were challenged using virulent ND 'chicken/Sudan/Obied/87' virus via an I/M route. Birds were observed up to 10 days, ND clinical signs or deaths were recorded.

2.6.2. Experiment II

In this experiment, the effect of the vaccine dose on immunity was determined on 2-weeks old commercial layer flocks. Fifty chicks were divided into five groups (G1, G2, G3, G4 and G5) of ten chicks each. Chicks in G1, G2, G3 and G4 were vaccinated via the I/N route with recommended dose, half of the dose and two and four times of recommended dose of the ND-I_2 vaccine respectively. Chicks in G5, were left unvaccinated as control. Four weeks later, all chicks were bled, and the serum was tested for ND virus antibodies by means of HI test; then, all chicks were challenged with the virulent ND 'chicken/Sudan/Obied/87' virus via an I/M route.

2.6.3. Experiment III

This experiment was conducted to determine the immunogenicity of the ND (I_2) vaccine under Sudan field conditions. Three flocks of commercial layers chickens were vaccinated at 3-weeks-old with ND- I_2 vaccine via I/O, I/N and D/W route. Another three flocks, at the same age were vaccinated with the recommended dose of ND-Komarov(K) strain I/N,I/O and D/W routes. Five flocks of adult indigenous village chickens were vaccinated with ND I_2 (I/O, I/N in D/W) and K-strain (I/N and D/W). The vaccinated flocks were observed for any post vaccination reactions. Four weeks later, all chicks were bled, and the serum was tested for ND virus antibodies by means of HI test.

2.7. Serological Test

2.7.1. Preparation of the Antigen

Ten fold serial dilutions of ND (LaSota strain) vaccine were prepared in PBS containing antibiotics mixture, each dilution was inoculated into an allantoic cavity of five 10-day-old embryonated chicken eggs, each egg received 0.1 ml of the inoculum . After 4 days of incubation at 37°C, allantoic fluid was harvested and kept at -20°C till used in HA and HI tests .The titre was calculated using the method of Reed and Muench (1938).

2.7.2. Haemagglutination (HA) and Haemaggultination Inhibition (HI) Tests

Using U-shape microtiter plate, two-fold virus dilutions were prepared in 0.025 ml of normal saline. Volumes of 0.025 ml of 1% suspension of chicken RBCs were added to each well, the test was read after 15 minutes incubation at room temperature.The HA titre was the reciprocal of the last dilution showing haemagglutination.The 4HA unit was used in HI test.

Beta procedure of HI test was used according to Chu (1960). Two fold serum(collected from each experiment) dilution were prepared as described for the virus above. Volume of 0.025 ml of the 4 HA units were added to each well. The plate was incubated for 15 minutes at room temperature for antigen/antibody reaction. Volume of 0.025 ml of 1% RBCs was added and the test was read after 30 minutes incubation at room temperature. The HI titre is the highest dilution causing complete inhibition of the 4HA unit of the virus.

2.7.3. Statistical Analysis

The statistical significance of differences between groups of data was determined using the two-tailed Student's unpaired t-test.

3. Results

3.1. Experiment-I

The mean HI-Ab titres and percent of protection produced by vaccination of two week-old chicks with ND-I_2 vaccine were shown in Table (1). I/O route gave significantly ($p < 0.01$)

higher antibody response compared to D/W and slightly better than I/N route. The mean HI titres and percent of protection produced were '5 log_2, 100%', '4.7 log_2, 90%' and '3.75 log_2, 45.5%' for I/O, I/N and D/W respectively

3.2. Experiment II

The mean HI-Ab titres and percent of protection produced by vaccination of two week- old chicks with ND-I_2 vaccine using recommended dose and half, 2 and 4 times of the recommended dose were shown in Table (2). The immunity produced and the percent of protection when using half of recommended dose were '4.375 log_2, 87.5%' compared to '5.5 log_2, 100%' and '5.7 log_2, 100%' for 2 and 4 times of the recommended dose respectively. Recommended dose produced HI titer of 4.83 log_2 and 90% protection.

3.3. Experiment III

The immunogenicity of the ND (I_2 and K) vaccines obtained from field trials were demonstrated in Tables (3 and 4). The mean HI-Ab titres of the vaccinated indigenus village chicken flocks by I_2 vaccine was 3.7 log_2, 6.0 log_2 and 5.0 log_2 for D/W, I/N and I/O routes respectively. While, the mean HI-Ab titres of the other two village flocks vaccinated by K vaccine were 6 log_2 for I/N route and 3.5 log_2 for D/W route.

Results of I_2 and K vaccination of commercial layers from different flocks showed that The immune response of commercial layer flocks was superior when ND- I_2 vaccine was used and the mean HI-Ab titres (log_2) among the three flocks vaccinated by the I_2 vaccine (I/O, I/N and D/W) were 8.5, 6.8 and 6.2 respectively. Compared to 3.6, 4.5 and 5.8 for Those received ND- K vaccine via I/O, I/N and D/W respectively (table 4).

Table (1). Challenge results of two weeks- old chicks received two doses of live ND thermostable (I_2) vaccine via three different routes.

Route of vaccination	Mean HI titre (log_2)					Protection level (%) one month post challange	
	Day pre-vaccination	14 days PV*		One month post 2nd dose		Vaccinated chicks	Control chicks
		Vaccinated chicks	Control chicks	Vaccinated chicks	Control chicks		
I/O	2	4.4	0.875	5	0.6	100	0
I/N	2	4.5	0.875	4.7	0.6	90	0
D/W	2	3.5	0.875	3.75	0.6	45	0

*PV: post vaccination.

Table (2). Challenge results of two weeks- old chicks received repeated doses of live ND thermostable (I_2) vaccine by I/N using different doses.

Dose of the vaccine	Mean HI titre (log_2)					Protection level (%) one month post challange	
	Day pre-vaccination	14 days PV*		One month post 2nd dose		Vaccinated chicks	Control chicks
		Vaccinated chicks	Control chicks	Vaccinated chicks	Control chicks		
Half recommended dose	2	4	0.875	4.375	0.6	87.5	0
Recommended dose	2	4.5	0.875	4.83	0.6	90	0
Two times recommended dose	2	5.1	0.875	5.5	0.6	100	0
Four times recommended dose	2	5.4	0.875	5.7	0.6	100	0

NB: Recommended dose: $10^{6.13}$ EID_{50}
*PV: post vaccination.

Table (3). The HI-Ab response of eight weeks local breed of chickens at Khartoum state vaccinated with NDV vaccines (I_2 and K) by different routes of administration.

Village name	Vaccine strain	Route	Mean HI titre (log_2)	
			Vaccinated chickens	Control chickens
Ezerga	I_2	D/W	3.7	0.33
Abuhalima	I_2	1/O	6	0.25
Dabba	I_2	1/N	5	0.5
Hassanya	K	1/N	6	0.33
ElGhaili	K	D/W	3.5	0.4

D/W: Drinking water
I/ N: Intranasal
I/ O: Intraocular

Table (4). The immunological response of six commercial layer flocks vaccinated with I_2 and Komarov vaccines as measured by HI test.

Vaccine strain	Total no. of chicks	No of sampled (sera)	Mean HI titre (log_2)	Percentage(%) protection(sera with 3 log_2 HI titre and above)
I_2	1200	35	8.5	100
I_2	3000	100	6.8	100
I_2	2500	80	6.2	100
K	1500	40	3.6	43
K	2000	38	4.5	75
K	1000	20	5.8	96

4. Discussion

Vaccination of young chickens which have high levels of maternally derived antibody (MDA) was previously considered as one of the main problems associated with control of ND in many parts of the world (Allan, 1972; Dawson and Allan, 1973). However, other researchers proved that passive immunity does not necessarily interfere appreciably with a vaccine applied by the nasal or ocular instillation and they recommend these routes for vaccinating young chicks having maternal immunity (Bornstein and Samberg, 1952). These routes were found to be effective in spite of insignificant maternal antibody at the time of vaccination. Conversely, passive and residual immunity was proved to neutralize efficiently a live virus vaccine applied intramuscularly and this route should not, therefore, be used in young chicks which have high levels of circulating antibodies (Komarov and Goldsmith, 1947).

In this investigation, better results were obtained when chicks were vaccinated via I/O route, resulting in high antibodies and protection. This agreed with the findings of vaccination trials conducted in other African countries, using the same or other thermostable vaccines of ND, such as Tanzania (Foster *et al.,* 1997), Mozambique (Alders and Fringe, 1998) and Zimbabwe (Anita, 1999). On the other hand, chicks vaccinated through the I/N route showed inferior immune responses and protection rates as compared to I/O but superior to D/W route. Similar findings were previously reported by kheir (1992) and Gaffar Elamin *et al.* (1993).

Based on these findings, the I/O route is recommended for the vaccine application especially when smaller flocks such as breeder replacement flocks and village backyard chickens have to be vaccinated. Following the virus administration into the bird conjunctiva, it reaches the upper respiratory tract through the naso-lacrymal duct where it multiplies luxuriantly to induce the required immune responses (Adwar and *Lukešová,2008)*. However, impracticability will supervene when large group numbers of broilers had to be immunized at once. Alternatively, mass vaccination methods such as D/W and spraying were to be encouraged it was believed that only that virus which comes in contact with nostrils and respiratory tissues around the pharynx can replicates successfully (Allan, 1971). Therefore, better immunity will not be expected to be stimulated by vaccine viruses applied via DW where the virus viability will be lost at the gastrointestinal tract (GIT), unless high amounts of NDV (> 10^5 EID_{50}) is contained in the vaccine (Shuaib *et al.,* 1985).

The effectiveness of different doses of ND-I_2 vaccine was also investigated during the present study. The results obtained revealed that at least double the recommended dose of the vaccine is required to offer total protection of chicks against challenge infection. Moreover, the vaccine was proved to be absolutely safe since double or four times the recommended dose resulted in no adverse vaccination reactions among vaccinated chicks which looked normal and healthy throughout the experimental course. Allan (1971) reported that, an effective immune response is dependent on the presence, in the bird, of an amount equivalent to $10^{9.5}$ EID_{50} infective particles. To achieve this, live virus vaccines that come in contact with the bird in concentrations not less than one five-hundredth of this amount must successfully invade the bird and multiply sufficiently to provide adequate immunity. In fully susceptible birds- according to the same author- this can be sufficiently achieved but when circulating antibodies- either passive or actively acquired- are present, they may inhibit the multiplication of the vaccine virus.

Usually mesogenic strains of NDV are used as booster vaccines because the mild live vaccines (those containing the lentogenic strains) do not multiply very efficiently when used as boosters in face of pre-existing immunity and hence secondary response that they induce is rarely very much higher than the primary response. This can be achieved by use of the more invasive live (intermediate) vaccines (Zakia *et al.,* 1983). This observation was valid during the experiments of this study where boosting resulted in little increase ($p< 0.01$) in HI titres when the I_2 was used for repeated vaccination. Similar observations were published by Rajeswar and Masillamony (1993) for LaSota and B1 and by Ali (1978) for Komarov vaccine.

Use of the thermostable vaccine of ND (I_2) was proved- form results of this work- very reliable, as it was safe, highly immunogenic and protective. It also gave protection and high immunity levels when used in the field for backyard and exotic chickens. It was even superior to the K strain, which is still used not only in Sudan but also throughout Africa, the

Middle East and Russia.

From the results obtained in the present experimental trials, levels of HI-Ab titres greater than 5.5 $\log_2$ are required to produce total protection after challenge.

It could be concluded that a vaccination programme of two weeks of age using 10^6 EID_{50} / dose of I_2 vaccine via I/O route followed by a booster dose after two weeks is recommended. As vaccination with K strain in remote villages areas may be difficult due to lack of cold storage chances, use of the I_2 thermostable vaccine is suggested.

References

[1] Adwar, T and *Lukešová, D. (2008). Evaluation of thermostable vaccines against Newcastle disease in village chicken used in tropics and subtropics.* Agricultura Tropica ET Subtropica*: 41(2) 74-79.*

[2] Alder, R.G. and Fringe, R. (1998). The use of thermostable Newcastle disease vaccine in Mozambique. In: *Improving Family Poultry Production in Africa*. Background information FAO.

[3] Alexander, D. J. (1991). Newcastle disease and other paramyxovirus infection. *Diseases of Poultry*. pp. 496-519. 9th edition. Iowa State University Press, Ames, Iowa, USA.

[4] Alexander, D. J. (2009).Ecology and epidemiology of Newcastle disease. In. *Avian influenza and Newcastle disease. A field and laboratory manual 1st edn*.. Edited by Capua, I and Alexander, D.J. Springer- Verlag Italia. Chapter 2 Pp 19-26.

[5] Alexander, D.J., Parsons, G. and Marshal, R. (1984). Infection of fowls with Newcastle disease virus by food contaminated with pigeon feces. *Veterinary Record* 115: 601-602.

[6] Ali, B.E. (1978). Response of chicks to vaccination with K strain of NDV under field conditions. *Indian Veterinary Journal* 55: 508-512.

[7] Allan, W. H. (1971). The problem of Newcastle disease. *Nature* (London) 234: 129-131.

[8] Allan, W. H. (1972). Newcastle disease control. *Agriculture* 79: 413-420.

[9] Allan, W. H., Lancaster, J.E. and Toth, B. (1978). Newcastle disease vaccines: their production and use. *FAO Animal Production,* Rome, Series No. 10 FAO.

[10] Anita, V. K. (1999). Results from Newcastle disease vaccine trials in Zimbabwe. *Improving Family Poultry Production in Africa*. Background information. FAO presented in Rabat Morocco, Feb 1999.

[11] Bensink, Z., Spradbrow, P.B. (1999). Newcastle disease virus strain I2--a prospective thermostable vaccine for use in developing countries. *Veterinary Microbiology* 16: 68 (1-2): 131-9.

[12] Bornstien, S., Arazi, A. R. and Samberg, Y. (1952). Some aspects of congenital passive immunity to Newcastle disease in chicks. The transfer of haemagglutination inhibitors from the maternal yolk to the chick. *American Veterinary Research Journal* 13: 373-378.

[13] Chu, H.P. (1960). A laboratory hand book on diagnosis of poultry disease. Rome, FAO. (Animal Health monogragp No. 2).

[14] Dawson, P.S. and Allan, W.H. (1973). The control of Newcastle disease by vaccinatio. In: *4th European Poultry Conference*. British Poultry Science Ltd. Edinburgh. 591-598, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, England.

[15] Foster, A., Chitukuro, H.R. and Tuppa, E. (1997). A field trial with thermostable V4 Newcastle disease vaccine in village in central Tanzania. *Proceedings in FPD Workshop*, M. Bour, Sengal Dec. 9-13. Issues in family poultry Research and Development.

[16] Gaffar Elamin, M.A., Khalafalla, A.I. and Ahmed, S.M. (1993). Observation on the use of Komarov strain of Newcastle disease vaccine in the Sudan. *Tropical Animal Health and Production* 25: 151-154.

[17] Kheir, S.A.M. (1992). Immune response to Newcastle disease vaccine (Komarov strain) given in drinking water. *The Sudan Journal of Veterinary Science and Animal Husbandry* 31: 14-19.

[18] Komarov, A. and Goldsmith, L. (1947). The use of live viruses in Palestine for the vaccination of poultry against Newcastle disease. *Cornell Veterinarian* 371: 368-372.

[19] Mahmood, M. Shahid; Siddique, F., Hussain, I., Ahmad, S.I. and Rafique, A. (2014): Thermostable vaccines for Newcastle disease: a review. World's Poultry Science Journal, Vol. 70, December 2014

[20] Meulemans, G. (1988). Control by vaccination. In: Alexander, D.J. (ed). Newcastle disease, pp. 318-332. Kluwer Academic Publisher, Boston, MA, USA.

[21] Murphy, F.A., Gibbs, E.P., Horzinek, M.C., and Suddert, M.J. (1999). Paramyxovirdae: *Veterinary Virology*, 412-428, third edition Academic Press USA.

[22] OIE (2012). Newcastle disease.. In Manual of diagnostic tests and vaccine for terrestrial animals .chapter 2-3-14 version updated in May 2012.

[23] Rajeswar, J.J. and Masilamony, P.R. (1993). Spray vaccine against ND. *Indian Veterinary Journal* 70: 404.

[24] Reed, L.J. and Muench. H. (1938). A simple method of estimating fifty percent end point. *American Journal of Hygiene,* 27: 493-497.

[25] Shuaib, M.A., Spalatin, J., McMillan, B. and Hanson, R.P. (1985). Studies on the development of a pelleted Newcastle disease virus (NDV) vaccine. *Vaccine* 3: 385-388.

[26] Spradbrow, P.B. (1992). Newcastle disease in village chickens. Control with thermostable oral vaccine. *Proceedings of an international workshop held in Kuala Lumpur*, Malaysia, 6-10 October, 1991. Pp. 189, ACIAR Proceedings No. 39.

[27] Wegdan, H.A., Khair, S.A.M., Bontsi, M. and Celia Abolink. (2010). Newcastle disease outbreaks in the Sudan from 2003 to 2006 were caused by viruses of genotype 5d. Virus genes 40: 106-110.

[28] Zakia, A.M., Ali, B.H., Mahasin, E. and Amal, M.M. (1983). The serological response of chicks to intranasal ND vaccination and re-vaccination. *Sudan Journal of Veterinary Research* 4: 99-103.