*The Sudan J. Vet. Res. (2003)**18:**15-24*
With 2 Tables in the text.

The Effect of Age of Chicken on the Immunogenicity and Protective Efficacy of the Newcastle Disease Thermostable Vaccine strain I_2

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ملخص البحث

تم تحضير بذرة التشغيل للعترة الأسترالية I_2 القابلة لتحمل درجات الحرارة العالية الواردة الينا من ACIAR، وتحديد عيارها في الأجنة وهو $10^{9.1}$ ج م ن / مل، ثم حقن اللقاح في أربع مجموعات عمرية من الكتاكيت عن طريق الأنف لمعرفة تأثير العمر علي الاستجابة المناعية والنسبة المئوية للمقدرة التحصينية بإعطائها جرعة لقاحية واحدة ($10^{6,1}$ ج م ن) أو مزدوجة بين الجرعة الأولى والثانية فترة أسبوعين في عمر يوم، أسبوع، أسبوعين أو ثلاثة أسابيع. وقد وضح من خلال التجربة أن الكتاكيت التي تم تحصينها عند عمر أسبوعين، وثلاثة أسابيع أعطت استجابة مناعية ونسبة حماية أفضل من التي تم إعطاءها اللقاح في عمر يوم وأسبوع. الكتاكيت التي أعطيت جرعة لقاحية إضافية أظهرت إرتفاعا في الاستجابة المناعية والنسبة المئوية للحماية مقارنة بالمجموعة التي أعطت جرعة لقاحية واحدة.

Summary

In the present study, a working seed of the thermostable NDV vaccine strain I_2 "supplied by ACIAR" was prepared in Sudan "CRVL, Khartoum". Its titre was determined in chick embryos and found to be $10^{9.1}$ EID_{50}/ml .The experimental trial was designed to determine the effect of age of chicks at the time of vaccination on the immunogenicity of the vaccine. Chicks were vaccinated once or twice intranasally with $10^{6.1}$ EID_{50} when they were one day, one week, two weeks and three weeks old. The results obtained indicated that vaccination of chicks at two and three weeks of age yielded better antibody (Ab) responses to the virus and higher protection rates. Boostering of chicks resulted in a relatively better ($P<0.05$) Ab responses in all groups of chicks.

Introduction

Newcastle disease virus (NDV) was first isolated in the Sudan from a natural outbreak and identified in 1962 (Karrar and Mustafa, 1964).The most prevalent strains of the virus belonge to the viscerotropic velogenic pathotype (Khalafalla *et al.*, 1992).

Vaccination against the disease was formerly practised in Sudan using imported and local vaccine strains. They included Komarov (K) strain (Karrar and Mustafa, 1964; Ali, 1978; Tabidi *et al*., 1998, Haroun and Hajer, 1989) and F strain (Mahasin *et*

al., 1980; Zakia *etal.*,1983). Despite extensive vaccination campaigns, ND outbreaks continued to occur in Sudan (Khalafalla *et al*., 2000) and worldwide (Spradbrow, 1993).

As live viral vaccines require a cold chain during transport, thermostable vaccines offered an opportunity to improve ND vaccination strategies for village poultry in Asia and Africa (Hanson and Spalatin, 1978; Kim and Spradbrow, 1978). They have been used successfully in some African countries such as Malawi (Sagild and Havesnape, 1987), Gambia (Jagne *et al*., 1991), Zambia (Alders *et al*., 1994), Cameroon (Bell *et al*., 1996),Tanzania (Foster *et al*., 1997) Mozambique (Alders and Fringe, 1998) and Zimbabwe (Anita, 1999) and some Asian countries such as Malaysia (Spradbrow, 1993; Spradbrow *et al*., 1997) and Vietnam (Tu *et al*., 1998).

This communication reports introduction of thermostable NDV vaccine strain I_2 into Sudan for the first time. It also reports on the immunogenicity of the vaccine to different age groups of chickens.

Materials and Methods

Viruses:

The viruses used were as follows:

i) Freeze-dried NDV I_2 vaccine seed, supplied by Prof. P.B. Spradbrow from the University of Queensland in Australia, was used to immunize chicks.

ii) A virulent NDV strain which was isolated from an outbreak of ND in El-Obeid, Sudan, with a titre of $10^{9.2}$ EID_{50}/ml, was used for challenge.

iii) Freeze-dried vials of 100-doses LaSota NDV vaccine strain were used for preparation of the antigen used in HA and HI tests.

Embryonated Chicken eggs:

Embryonated eggs were obtained from Coral Poultry Company, Sudan, and incubated at 37^{o}C with a relative humidity of 60-70% before being inoculated with the virus at the age of 9-10 days of incubation.

Experimental chicks:

A total of one-hundred, one-day-old, male Bovan chicks were used in this study. They were obtained from Coral Poultry Company farm, Khartoum, Sudan. The chicks were divided into groups and reared in special metal cages until they reached the required age. They

were vaccinated against infectious bursal disease (IBD) at two-weeks of age.

Blood sampling:

Blood samples were collected from chicks used in vaccination trials by heart puncture, left overnight at room temperature to clot and then centrifuged at 1000 rpm for 10 minutes.Separated sera were stored at –20^{o}C till used.

Inoculation of embryonated eggs:

The working seed and the experimental batch of NDV vaccine strain I_2 were prepared by inoculation of 9-11 day-old embryonated chicken eggs. Embryonated eggs with viable embryos were swabbed with 70% alcohol and inoculated by the allantoic route with sterile disposable 1 ml syringes. Each egg received 0.1 ml of the inoculum and the pore was sealed with melted paraffin wax. Eggs were then incubated at 37^{o}C and candled daily for four consecutive days to check for embryos death. Embryos that died during 24 hrs of inoculation were regarded as non-specific and discarded. At four days post inoculation (p.i), all eggs were chilled at 4^{o}C for at least two hrs before the allantoic fluid was aseptically collected into sterile vials.

Preparation of master and working seeds and I_2 vaccine:

One freeze-dried ampoule of NDV strain I_2 was diluted with 20ml PBS and divided into ten vials, two ml each, and stored as master seed at –20^{o}C.Working seed was prepared in egg embryos from the master seed by inoculation of 0.1 ml /embryonated egg via the allantoic route.

Two vials of the working seed were thoroughly mixed and used for inoculation of 40 embryonated eggs by the allantoic route with a dose of 0.1ml/egg as described above. Each egg was harvested separately and the allantoic fluids (AFs) were tested by the HA test for the virus growth. Positive AFs were then pooled, centrifuged at 2000 rpm for 30 minutes, dispensed in 2ml cryogenic vials and stored at –20^{o}C prior to use as vaccine.

Titration of the vaccinal virus:

Ten-fold serial dilutions of the experimental batch of the vaccine were prepared in normal saline (NS) and each dilution was inoculated into five, 9-10 day-old chicken embryos via the allantoic route (0.1ml/embryo). These embryonated eggs were candled daily. The AF was harvested on day 4p.i. and tested for virus growth by HA

and HI tests.The titre was then calculated using the method of Reed and Muench (1938).

Haemagglutination (HA) and haemagglutination inhibition (HI) tests:

To prepare red blood cells (RBCs) used in HA and HI tests, blood was collected into an equal volume of Alsever's solution by heart puncture of healthy 3-4 week-old chicken.The blood was then clarified by centrifugation at 1000 rpm for ten minutes.The supernatant was discarded and an equal volume of sterile PBS was added to packed RBCs. The RBCs were washed three times and packed cells were diluted to 1% for use in HA and HI tests.

For HA test, volumes of 0.025ml PBS were placed in all wells of U-shaped microtitre plates and two-fold serial dilutions of the virus were made. A volume of 0.025ml of 1% suspension of RBCs was then added to each well.The plates were incubated at room temperature for 30 minutes and agglutination of RBCs was observed. The HA titre was calculated as the reciprocal of the last dilution showing haemagglutination.

For HI test, the Beta procedure was employed. A constant virus dilution with serially diluted sera was used according to Chu (1960). Two-fold serial dilutions of the test sera were prepared in PBS in 0.025ml volumes in microtitre plates. A volume of 0.025 ml of the virus antigen containing 4HA unit, was added to each well. The plates were then left for 30 minutes at room temperature. Volumes of 0.025ml of 1% RBCs suspension were added to all wells. The test was read after 30 minutes of incubation at room temperature. The serum HI titre was the reciprocal of the highest serum dilution that inhibited haemagglutination of chicken RBCs by 4HA units of the virus. Controls included one row containing hyper-immune serum (positive control), one row containing serial dilutions of the antigen to confirm the 4HA units and one row containing RBCs (reagent control).

Preparation of 4HA units of the virus:

Undiluted virus suspension was titrated by HA test. The last well showing HA was considered to contain one HA unit and accordingly, the 4HA units were calculated. The virus suspension was then diluted to contain 4HA units per 0.025ml.

Challenge infection:

Vaccinated and control chicks were challenged with NDV ElObeid strain. All chickens were challenged with 0.2ml of a virus suspension containing $2x10^{8.2}$ EID_{50}/0.1ml using I/M route. Challenged chickens were thereafter observed daily for a period of 14 days after the challenge. The mortalities and clinical signs were recorded and PM examination was done on all dead birds.

Experimental design:

One hundred, one-day-old chicks were divided into five groups of 20 birds each.Chicks of the first four groups were vaccinated intranasally at one day-old and 1^{st}, 2^{nd} and 3^{rd} weeks of age, respectively, with 0.1 ml of I_2 vaccine containing $10^{6.1}$ EID_{50}. The fifth group was left as unvaccinated control. Vaccinated chicks were further divided into two subgroups of 10 chicks each (a & b). Chicks in the subgroup (a) were not boostered while chicks in the subgroup (b) received a booster dose of the same vaccine 2 weeks following the first vaccination. All five groups including the control were challenged four weeks for booster dosed group and six weeks for (single dosed group) post last vaccination by 0.2 ml of the field NDV containing $2x10^{8.2}$ EID_{50}/0.1 ml I/M. Pre-and post vaccination serum samples were collected. The antibody response to vaccination was assessed by the HI test.

Statistical analysis:

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

Results

The titre of the vaccine was estimated at $10^{9.1}$ EID_{50}/ml, ie the same as indicated in the manufacturer guideline for the vaccine production. The prepared vaccine was confirmed to be free from viral, bacterial, mycoplasmal and fungal contamination.

Pre-and post vaccination mean HI titres (log_2) and percentage protection among the four groups vaccinated are shown in table 1. The Ab response and percentage protection among the four subgroups following the administration of the booster dose are shown in table 2. Vaccination of 2 and 3-weeks-old chicks gave comparable immune responses and percentage protection against challenge. The percentage protection among the controls was zero in all age groups throughout the experimental course. The booster dose produced a relatively better

Ab response in all chicks.

Table 1: The HI–Ab response and percentage protection following challenge of chicks intranasally vaccinated with a single dose of NDV I_2 vaccine strain.

	Mean HI	6 weeks after vaccination		Protection %	
age	titre before vaccination (log_2)	Vaccinated chicks	Control chicks	Vaccinated chicks	Control chicks
One day	5	3.88	1	50	0
One week	4	4.1	1.6	60	0
Two weeks	2.375	4.5	0.166	82	0
Three weeks	1.275	4.55	(zero) 0	82	0

Table 2: The HI-Ab response and percentage protection following challenge of chicks vaccinated and boostered with a dose of NDV I_2 vaccine strain.

	Mean HI titre before	2 weeks after first vaccination		4 weeks after booster dose		Protection %	
Age	vaccinati-on (log_2)	Vaccin-ated	Con-trol	Vaccin-ated	Con-trol	Vaccin-ated	control
One day	5	3.77	2	4	1	60	0
One week	4	4.2	1.9	4.4	1.6	63.7	0
Two weeks	2.375	4.2	1.1	4.621	0.166	91	0
Three weeks	1.375	4.28	0.4	4.71	0	91	0

Discussion

Vaccination failure is encountered in most cases when the kind of the vaccine used is not the right one especially in matters pertaining to birds. The inappropriate timing constitutes a major factor in vaccination failure as well. In fact, the real practical problem is how to provide the right kind of vaccine to thousands of backyard chickens scattered in the fields and villages throughout a vast country.The vaccine intended for use should suit the adverse environmental conditions, mainly high temperature and lack of cold storage facilities.

Experimental trials to determine the effect of age of chickens on the protective efficacy of the vaccine were conducted. Results obtained showed that commercial layer chicks produced in Khartoum, Sudan had high levels of maternally derived antibodies (MDA) to NDV. The effect of MDA on the immune response elicited from ND vaccination is well documented (Borland *et al*., 1980; Mahasin *et al*., 1980; Westbury *et al.*, 1984; Haroun and Hajer, 1989). It is also believed that satisfactory levels of immunity are not reached after vaccination of maternally immune chicks unless vaccination is delayed until the 14^{th} –21^{st} days of life (Allan, 1971). On the other hand, chicks obtained from unvaccinated flocks could be adequately protected by vaccinating them at first day of age (Keeble and Wade, 1963). Conversely, the presence of MDA was found to reduce the level of protection produced by vaccination (Allan, 1973; Parry and Aitken, 1977). Unfortunately, MDA may not be always protective against natural infection even in moderate or high titres (Allan, 1973). It was also believed, however, that inadequate immune response to early vaccination was due to the fact that the immunological maturity in chickens was only reached at the fifth week of age (Wolfe and Dilks, 1948). In this study, chicks vaccinated with the I_2 ND thermostable vaccine administered intranasally at one and seven days of age gave low HI antibody titres and weak protection as compared with that given to the older birds.There was a clear decrease in the mean antibody level after vaccination of one-day old chicks while slight increase ($P<0.05$) was noticed among the one week old ones, which was considered to be insignificant. This indicates that even moderate levels of MDA interfere with the immune response to vaccination at early life.These findings are clearly substantiated by, and in agreement with, the aforementioned reports. However, boostering of these chicks two weeks after initial vaccination resulted in a slight increase ($P<0.05$) in the HI titre with moderate levels of protection.

Initial vaccination of birds with low levels of MDA at the age of two and three weeks resulted in higher levels of HI antibody titre and better protection. The immune response of these birds, though more enhanced than that of younger chicks, was not satisfactory. Even the booster dose given two weeks after the first one resulted in slight increase in titres in the two age groups with a better though not

complete protection upon challenge. It should be concluded, however, that initial vaccination of chicks at the age of two and three weeks results in relatively comparable and better results.This may be attributed to waning of MDA as well as progress of the immunological competence with age.

As initial vaccination is required at as early age as possible in ND endemic areas and in areas where a virulent field virus is prevalent we assign the age of two weeks as the most reliable age for vaccinating chickens against ND in Sudan.This assignment was emphasized by the fact that the percentage protection of the control groups was zero throughout the experimental trials.

Acknowledgements

The authors are very much grateful to Prof. P.B. Spradbrow from the University of Queensland in Australia and to Dr, Sobhi, A.M. Kheir, Department of Virology, CRVL (Sudan) for their kind provision of the thermostable vaccine strain and the virulent field virus isolate, respectively.

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