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Relationship between antibody titers from V_4-in-cassava vaccination and protection against velogenic virus challenge in laboratory chickens

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Unvaccinated laboratory-raised, five week old cockerels were fed V_4 newcastle disease vaccine in cassava once, twice or thrice; and tested for hemagglutination inhibition (HI) antibody response. They were subsequently challenged with a local isolate of velogenic newcastle disease virus (NDV). Immune status of the chicken flocks improved with number of vaccinations. Survival from velogenic virus challenge varied significantly with pre-challenge HI antibody titer, protection being better at $\log_2$ titer of ≥3 than at $\log_2$ titers of ≤ 2 but no difference was observed between titers of 3, 4 and 5. Although HI $\log_2$ antibody titers of 3 and 4 were apparently observed to protect vaccinated chickens against ND mortality in this work, 7 out of 8 (87.5%) of the chickens that manifested torticollis (neurological disease symptom) had pre-challenge HI antibody titers within this range. Thus, it is suggested that while $\log_2$ HI antibody titer of 3 may be taken as cut-off point for sero-conversion, titers of 5 and above may protect against neurological symptoms.

Key words: V4, cassava-based vaccination, velogenic challenge, HI antibody titers, chicken protection.

INTRODUCTION

Protection of chickens against newcastle disease (ND) by vaccination has been practiced for over half a century, particularly for intensely reared commercial poultry. Both live attenuated and inactivated virus vaccines have been used but the former is considered advantageous on account of the ease of administration, low dosage required and herd immunity through cross-infection of contact chickens by vaccinated birds excreting vaccine virus. The V_4 vaccine has an added advantage of thermostability, thus reducing the need for cold-chain storage, and being amenable to oral delivery through feed (Ideris et al., 1987a, b; Samuel et al., 1992). This makes it the vaccine of choice for village chicken flocks with their free range life style. In such vaccination programs, immune status in terms of level of protection achieved, needs to be determined in order to estimate the number and regularity of vaccinations to be administered. Determination of antibody titers has always been used for this purpose, even though humoral antibodies do not seem to be the only protective mechanism involved. Indeed, there is a lack of correlation between circulating antibody titers and resistance to infection; and both cell mediated immunity and local immunity in the respiratory tract are the other suggested protective mechanisms (Ibrahim et al., 1980). Winterfield et al. (1980) also observed that a positive neutralization and HI response may not necessarily be indicative of a capacity to protect against virulent NDV. So far, neither the assay of cell mediated immunity nor that

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of local immunity in the respiratory tract is as convenient as the assay for antibodies to be applied routinely and for a large number of samples. Hence, determination of serum antibody titers continues to be the choice in evaluating immune status of chickens against ND; and to that effect, HI test method remains the most simple. However, the relationship between HI antibody level and protection seems to depend on a number of factors, namely, the vaccine strain, the route of administration and the virulence of the challenge virus. Allan and Gough (1974) observed that La Sota vaccine is associated with a higher death rate than B1 vaccine at comparable HI antibody levels, thus confirming that HI antibody/velogenic virus challenge relationship may vary between strains. However, death rate appears to be inversely related to the flock HI antibody levels; and $\log_2$ HI antibody titer of 3 has long been widely accepted as protective against ND mortality (Allan and Gough, 1974; Spradbrow et al., 1980; Jagne et al., 1991). Here protection is defined by survival but other factors may define the totality of protection, including immunity from residual clinical disease, such as torticollis, and no-reduction in egg production.

In this paper, analysis of variance is used to determine relationship between HI antibody titers of chickens vaccinated with V_4 through cassava feed and total protection afforded against challenge with a local (Nigerian) NDV strain isolated from a deceased guinea fowl.

MATERIALS AND METHODS

Vaccine

Freeze-dried sample of V_4-UMP-NDV was obtained from Professor Aini Ideris of the Faculty of Veterinary Medicine and Animal Sciences, University Partanian, Malaysia. The virus sample was purified by the limiting dilution method, described by Ideris et al. (1987); and re-selected for thermostability at 56°C as described by Iroegbu and Nchinda (1999). Freeze-dried ampoules of the purified, heat-selected (thermostable) V_4-UMP-NDV vaccine were prepared from allantoic fluid harvests at the National Veterinary Research Institute (NVRI), Vom, Plateau State Nigeria, and stored as stock.

Chickens and cassava-based vaccination

Unvaccinated day-old cockerels were purchased for the experiment from Arroma Farms Ltd., Awka, Anambra State, Nigeria. They were reared in laboratory poultry cages on commercial feed formulation (Guinea Feeds, Benin City, Nigeria) and were used for the experiment at 5 weeks of age. Chickens to be feed-vaccinated were starved for about 18 h and thereafter 500 g of vaccine-coated cassava feed was given to the 23 or 22 chickens in a vaccination room. After all the feed had been consumed or each chicken estimated to have eaten an average of 20 g, the room was thoroughly cleaned and the unvaccinated contact chickens were introduced. Unvaccinated control chickens were isolated in a separate room. All chickens, including the in-contacts and controls, were bled by wing vein puncture (before each vaccine feeding at three-week intervals) and tested for serum HI antibodies. Cloacal swabs were taken in 2 mL of virus transport medium, 6-10 days after exposure to vaccine virus, and cultured in 10-day embryonated eggs to determine which vaccinated and in-contact chickens were excreting the virus.

Velogenic virus challenge

Vaccine-fed, in-contact and unvaccinated control chickens were all moved to an infection room and left with normal feeding for two weeks to acclimatize before challenge; this was one week after the last vaccine feeding or at 12 weeks of age. The chickens were then starved for 18 h and subsequently allowed to drink water seeded with $\approx 10^5$ EID_{50}/ml of velogenic (neurotropic) NDV obtained from the National Veterinary Research Institute (NVRI), Vom, Plateau State Nigeria. Thus, all the birds were challenged at the age of 14 weeks. The body temperatures and weights were monitored before and throughout challenge period as indicators of clinical illness. Velogenic virus isolation was attempted with cloacal and tracheal swabs at day 3 post-challenge, as well as with pathological specimens- spleen, kidney, proventriculus and bursa- from chickens that presumably died of the challenge.

Assay of serum antibody

Serum antibody was assayed using a modification of the micro-titration HI test method described by Allan and Gough (1974). Two-fold dilutions of the sera (one sample in a row) were made in a 96-well U-bottomed micro-titer plates using an 8-channel micro-pipetter (Dyna-Tech) set to deliver 50 µl. Serum dilution stopped at the 10^{th} well; the 11^{th} and 12^{th}, each containing 50 µl of diluent (PBS), were antigen and cell control wells, respectively. Next, 50 µl of 4HA units of antigen was added to all wells, except the cell control well, and the plates incubated at room temperature for 20 min. Finally, 50 µl of 1% washed red blood cell (RBC), obtained from 3-week old chick, was added to all wells. The volume of reactant mixture in the cell (RBC) control well was made up to 150 µl by addition of extra 50 µl of PBS. Incubation was at +4°C for 45 min after gentle tapping of the plates at the sides to mix and re-suspend the RBCs. A magnifying mirror was used to read the test and the inhibition endpoint taken as the well in which the RBC button was reduced to half the size of that in the RBC control well. Results were recorded as $\log_2$ of the reciprocal of serum dilution giving 50% hemagglutination. Positive NDV antiserum, obtained from the National Veterinary Research Institute (NVRI), Vom, was included as standard.

RESULTS

A total of 194 chickens- 67 feed-vaccinated, 77 in-contacts and 50 unvaccinated controls - were used in the experiments in which chickens were exposed (vaccine-fed or in contact with the vaccinated) to vaccine virus once, twice or thrice, respectively. Intervals between exposures were 3 weeks each.

Proportion of chickens which excreted vaccine virus 6-10 days after a single exposure varied from 42.9% among the in-contacts to 76.2% among the vaccine-fed. Approximately 74.0% of all the chickens excreted velogenic virus following challenge.

Table 1 shows the distribution of chickens with detectable HI antibodies and mortality/survival at challenge according to number of exposures to vaccine virus coated on to cassava feed. The highest proportion of chickens with $\log_2$ HI antibodies titers of ≥3 (86.4%)

Table 1. Distribution of chickens attaining HI $Log_2 \geq 3$ antibody titers and mortality after 0-3 feeding with vaccine-coated cassava meal.

Number of times chickens were fed with vaccine-coated cassava meal	No. of chickens given treatment	No. (%) chickens with of HI log_2 ≥3 antibodies titer	No. (%) dead at challenge	No.(%) of chickens that survived challenge
0	50	0 (0.0)	49 (98.0)	1(2.0)
1	23	4(17.4)	12 (52.2)	11(47.8)
2	22	19 (86.4)	4 (18.2)	18(81.8)
3	22	13 (59.1)	0 (0.0)	22 (100.0)
Total	117	36 (30.8)	65 (55.6)	52 (44.4)

Table 2. Frequency distribution of pre-challenge serum antibody titers and mortality following velogenic virus challenge among vaccinated chickens, in-contacts and unvaccinated controls.

Experimental Chicken Group	Number of chickens	No(%) chickens with HI log_2 Antibody Titer						Percent mortality per chicken group
		<1	2	3	4	5	>5	
Vaccinated	67	**19(28.4)**	**12(17.9)**	**14(20.9)**	**4(6.0)**	**7(10.4)**	**11(16.4)**	**16 (23.9)**
In-contacts	77	25 (32.5)	15 (19.5)	16 (20.8)	11 (14.3)	3 (3.9)	7 (9.1)	**36 (46.8)**
Unvaccinated controls	50	50 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**49 (98.0)**
Total	194	94(48.5)	27(13.9)	30(15.5)	15(7.7)	10(5.1)	18(9.3)	
Mortality(%) per Antibody Titer		82 (92.1)	16 (66.7)	1 (3.0)	2 (12.5)	0 (0.0)	0 (0.0)	101 (52.1)

appeared among the twice vaccinated while only 59.1% of the thrice vaccinated chickens achieved this level of response. However, all the thrice vaccinated chickens (vaccine-fed and in-contacts) survived velogenic virus challenge. Survival rate among chickens that were twice exposed to V_4-virus was 81.8% while 47.8% of those exposed once survived. Only 1 out of the 50 unvaccinated control chickens (2%) survived velogenic virus challenge.

Frequency distribution of pre-challenge serum antibody titers and mortality at challenge among the vaccinated, in-contacts and unvaccinated control chickens is shown in Table 2. Higher proportion of the directly vaccine-fed chickens (71.6%) had detectable HI antibody when compared with 67.5% of in-contacts but the difference is not significant ($P>0.05$). Mortality was 98.0% among the unvaccinated controls when compared with 23.9% among the directly vaccine-fed chickens and 46.8% among the in-contacts. Mortality distribution according to antibody titers varied from 92.1% for chickens without detectable serum antibody to 66.7% for those with log_2 HI antibody titer of 2, 3% for titer of 3 and 12.5% for titer of 4. All chickens with log_2 HI antibody titers of 5 and above survived velogenic NDV challenge.

The challenge with velogenic NDV produced four broad clinical responses, namely, subclinical infection in 38 chickens, clinical illness with complete recovery in 7, clinical illness with residual paralysis and torticollis in 8, and death in 17. The sub-clinically infected chickens of challenge while the body temperatures remained within showed an average weight gain throughout the duration the normal range (41.4 - 41.8°C). The birds, which were initially sick but fully recovered subsequently, at first showed decrease in body weight during the period of clinical illness (Figure 1). This coincided with rise in body temperature above 42.5°C on the 8^{th} day post-challenge (Figure 2). Thereafter, these convalescent birds showed steady weight gain. For the survivors with residual torticollis, there was apparent weight loss throughout the period of challenge (Figure 1); the body temperatures, also, increased up to 42.4°C on the 8^{th} day post-challenge before declining (Figure 2). The birds which died following challenge showed a rapid decline in average body weight (Figure 1) and conversely sharp rise in body temperature before death on the 6^{th} day post-challenge (Figure 2). That the chickens died of ND was confirmed by isolation of velogenic NDV from deceased chickens in addition to the characteristic ND signs and pathology observed.

The distribution of these responses in chickens according to pre-challenge serum antibody titers is shown in Table 3. Analysis of variance was used to show that a significant difference existed ($F_{cal} = 40.4$; $P < 0.01$) between antibody titers and number of chickens which survived challenge (Table 4). By comparing the differences in protection rate achieved at HI log_2 antibody titers ranging from 0 to 5 using Fisher's least significant difference (F-LSD), it was demonstrated that the number of chickens surviving challenge varied significantly with pre-challenge HI antibody titers (Table 5). The level of protection given by log_2 HI antibody titer of 3 was significantly different from that of zero and 2; and the

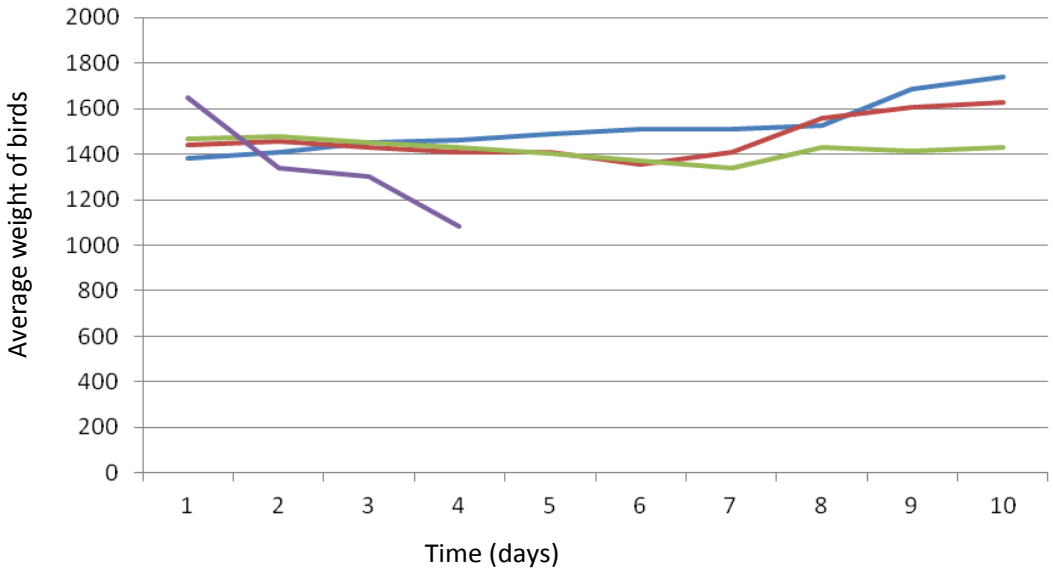

Figure 1. Changes in average body weights among chickens manifesting different clinical responses to velogenic newcastle Disease Virus challenge- chickens with subclinical infection ( ); chicken that completely recovered from clinical illness ( ); chickens that survived challenge with residual paralysis ( ); and chickens that died of the challenge virus infection ( ).

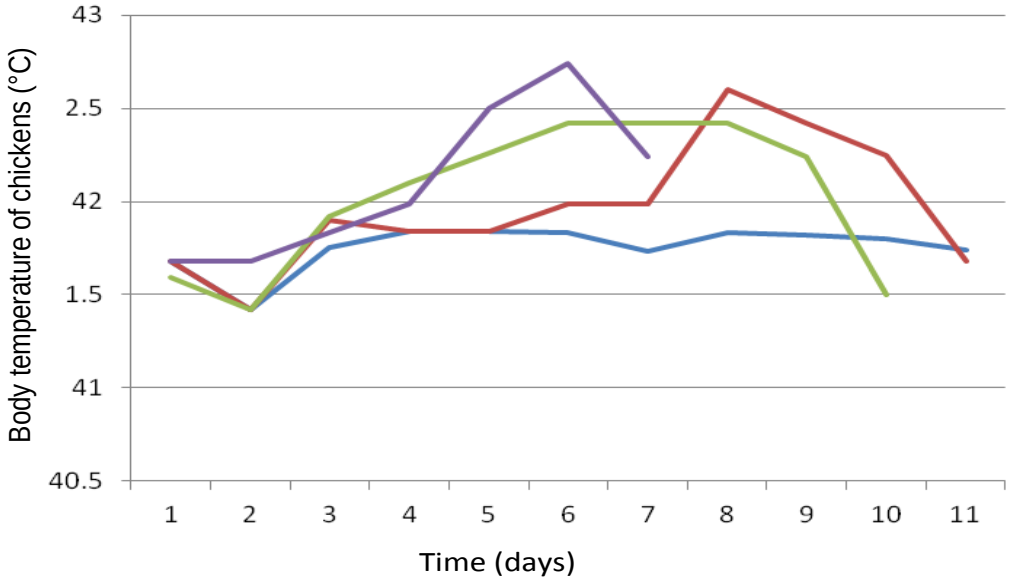

Figure 2. Body temperature changes in challenged with velogenic newcastle Disease Virus and showing different clinical responses - subclinical infection ( ); complete recovery from clinical illness ( ); residual paralysis or torticollis ( ); and death ( ).

protection achieved by $\log_2$ HI antibody titer of 3 was significantly lower than those of titers 4 and 5. However, protection achieved with $\log_2$ HI antibody titers of 4 and 5 (represented by absence of mortality) were not significantly different from the protection given by titer of 3. Seven out of the 8 (87.5%) chickens with torticollis had pre-challenge antibody titers of 3 or 4 (Table 3).

DISCUSSION

Establishment of clinical infection in birds challenged with velogenic NDV was inferred from decrease in body weight as well as rise in body temperature above 42°C Recovery from clinical illness with or without residual neurological symptoms (or torticollis) was marked by

Table 3. Distribution of reactions of chickens to velogenic virus infection (challenge) in relation to serum antibody level.

HI Log_2 titer range	Number (%) chicken in each reaction type (N = 70)				Total
	Subclinical infection (health survivor)	Clinical illness (totally recovered survivor)	Clinical illness (residual paralysis, torticollis)	Death	
<1 – 2	4 (5.7)	3 (4.3)	1 (1.4)	16 (22.9)	24 (34.3)
3 - 5	25 (35.7)	4 (5.7)	7 (10.0)	1 (1.4)	37 (52.9)
6 – 8	8 (11.4)	0 (0.0)	0 (0.0)	0 (0.0)	8 (11.4)
>8	1 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)
Total	38 (54.3)	7 (10.0)	8 (11.4)	17 (24.3)	70 (100.0)

Table 4. Analysis of variance (ANOVA) of number of surviving chickens (%) with five different pre-challenge HI log_2 titers (1 - 5) following challenge with velogenic NDV.

SV	df	SS	MS	F_{cal}	Probability
HI log_2 titer	4	129353	32338.25	40.5**	<0.01
Error	108	86222.22	798.35	–	–
Total	112	215575.22	–	–	–

decline in the previously raised body temperature; and improvement in body weight only for those without torticollis. Persistent decline in body weight among those with torticollis resulted from starvation on account of their inability to peck food. The challenged birds which remained apparently healthy or had sub-clinical infection exhibited steady rise in body weights and normal body temperatures.

Excretion of virus (both V_4 and velogenic NDV) confirmed establishment of infection by ingested virus. The general rise in frequency of high antibody titers with number of exposures to vaccine virus was expected. It is difficult to explain the non-significant but higher proportion of birds with HI log_2 ≥3 antibody titers among twice vaccinated (86.4%) than the thrice vaccinated (59.1%); it may have arisen from the fact that different chickens ingested remarkably varied quantities of vaccine-coated feed. Samuel et al. (1992) killed chickens of similar age and size soon after mock feed-vaccination with paddy rice. The rice contents of the crop varied from 0.4 to 16 g with a mean of 8.6 g. The considerable variation in intake of vaccine-coated feed implies that not every chicken may get adequate dose of the vaccine virus to stimulate high titer antibody response. All the same, three vaccinations gave superior protection over one or two.

The significant superiority in chicken protection achieved at log_2 HI antibody titers of 3 and above and the non-significant difference between titers 3, 4 and 5 led to the adoption of HI log_2 titer of 3 as the cut-off point of sero-conversion. This adoption is consistent with the observations and conclusions of Spradbrow et al. (1980) and Jagne et al. (1990). It has been observed, however, that herd immunity (arising from infection of other flock with vaccine virus excreted by vaccinated chickens), which is a very desirable outcome of a vaccination program, would only be achieved with newcastle disease when more than 85% of the flock have HI log_2 antibody titers of >3 after two vaccinations (van Boven et al., 2008). Based on field observations where 66% of researched flock succumbed to velogenic NDV challenge with HI log_2 titers less than 4, it has been suggested that only birds with titers greater than 4 after multiple vaccinations will survive similar challenge (Kapczynski and King, 2005; Kapczynski et al., 2013) However, the log_2 HI antibody titer of 5 and above taken by Phillips (1973) and Allan et al. (1978) as protective against newcastle disease, apparently conferred 100% protection in this work with none of the chickens having such antibody titers suffering torticollis. Hence, log_2 HI antibody titer of 5 may generally be aimed at, particularly when considering how many vaccinations would be required to protect free range village chicken flocks.

Both categories of vaccinated chickens (directly vaccine fed and in-contacts) with or without detectable serum antibodies survived the challenge to varying degrees while all the unvaccinated controls died on challenge with the exception of one. Therefore, as long as a chicken has been exposed to vaccine, absence of detectable serum antibody may not always signify lack of immunity against newcastle disease (Johnston et al., 1992). Indeed, immune mechanisms other than humoral, e. g., cell mediated immunity, may contribute to protection against newcastle disease (Ibrahim et al., 1980; Kapczynski et al., 2013). The significance of predominance of torticollis among chickens with serum antibody titers of 3 or 4 (10.0%) is not easily discernible but may suggest immune-complex

Table 5. Differences between survivor means in chickens with five different pre-challenge HI log_2 titers.

HI log_2 titer means	<1	2	4	3	5
	5.55	**22.2**	**83.3**	**90.0**	**100**
100.0	94.45*	77.8*	16.7^{ns}	10.0^{ns}	–
90.0	84.45*	67.8*	6.70^{ns}	–	
83.3	77.75*	61.1*	–		
22.2	16.65*	–			
5.55	–				
	$F\text{-}LSD_{0.01}(X_5\text{-}X_3)$ =32.86		$F\text{-}LSD_{0.01}(X_2\text{-}X_0)$ =17.75		
	$F\text{-}LSD_{0.01}(X_3\text{-}X_4)$ =34.40		$F\text{-}LSD_{0.01}(X_5\text{-}X_0)$ =26.40		
	$F\text{-}LSD_{0.01}(X_3\text{-}X_0)$ =25.50		$F\text{-}LSD_{0.01}(X_5\text{-}X_2)$ =29.70		
	$F\text{-}LSD_{0.01}(X_3\text{-}X_2)$ =26.29		$F\text{-}LSD_{0.01}(X_4\text{-}X_2)$ =31.43		
	$F\text{-}LSD_{0.01}(X_5\text{-}X_4)$ =37.00		$F\text{-}LSD_{0.01}(X_4\text{-}X_0)$ =28.13		

*Statistically significant (P = 0.01). ^{ns}Non-significant (P = 0.01).

disease. However, it further underscores the apparent advantage of HI log_2 titers of 5 and above in protection against neurological disease. It is noteworthy, though, that the strain of newcastle disease virus used for the challenge was a velogenic neurotropic pathotype (Echeonwu et al., 1993), which invades the central nervous system causing, among other pathologies, neurological degeneration and, particularly, destructive lesions in the hypothalamus (Auer, 1952). Thus, HI log_2 titers of 5 and above may protect against viral invasion of the CNS.

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