



Formalin Inactivated Infectious Bursal Disease Virus Vaccine Immunogenicity in Indigenous Chickens in Kenya

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Abstract

Background: Infectious bursal disease vaccination failure and subsequent outbreaks in vaccinated chickens are a challenge in poultry production. This could be due to use of live vaccines which may revert to virulence resulting in disease. Further, live vaccines may become non-viable due to poor handling and yield no immune response. Killed vaccines developed using indigenous strains could offer solutions to the challenge. Immunogenicity of five formalin inactivated virus isolates from field outbreaks in Kenya were determined. The isolates, designated as E3, E9, E19, E34 and, E42, were prepared at 10⁴EID₅₀ and each inoculated into six 4 week old specific antigen negative (SAN) indigenous chicks. 0.3mls was administered intramuscularly at day 0, 14 and 21 and titres levels measured at inoculation (baseline), days 14, 21, 28 and 35.

Results: Immune responses were detected by Agar Gel Precipitation Test (AGPT) and Enzyme Linked Immunosorbent Assay (ELISA). All the isolates elicited detectable immune response by day 14. Antibody titre values by day 21 were above 396 and considered positive. Highest titre value (9140) was recorded on day 28 in response to E19. Titres variations between isolates were not statistically significant (P=0.9639).

Conclusions: All isolates were immunogenic. Isolates E3 and E19 consistently yielded high titres and were recommended as most suitable for development for use in a vaccination regimen.

Keywords: Infectious bursal disease, immune response, formalin killed virus, indigenous chickens

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Introduction

Infectious bursal disease (IBD) is an immunosuppressive disease of young chickens caused by Infectious bursal disease virus (IBDV). Affected chickens show signs of anorexia, depression, severe prostration, and death. Lesions on dead chickens include dehydration, hemorrhages in the leg and thigh muscles, urate deposits in kidneys and enlargement of the bursa of Fabricius¹.

Recovered birds tend to have retarded growth and secondary infections due to immunosuppression. Infectious bursal disease virus is worldwide in distribution and is an important factor wherever chickens are produced including Africa. IBD is ranked the second most important disease affecting productivity in poultry farms in Kenya² after Newcastle disease. Strict hygienic conditions within poultry farms, coupled with vaccination, are used as control and preventive measures of the disease^{3,4}. However, most indigenous chickens in Kenya are farmed at sectors 3 and 4 of the FAO classification of poultry production systems, where bio-security level is low⁵. This therefore makes control of the disease by vaccination critical for effective protection of indigenous chickens in Kenya. However, like in many other parts of the world, instances of vaccination failure occur in Kenya⁶.

Factors that determine generation of protective immune responses are either factors of the host, vaccinator or vaccine. Examples of host factors are breed, passive immunity and age; those of vaccinator include dilution of the vaccine and handling including maintenance of cold chain; while those of the vaccine include the type of vaccine, antigenic relatedness with challenge strain and immunogenicity.

Classical live vaccines achieve protection but possess residual pathogenicity with the potential risk of reversion to virulence⁷. Vaccines classified

as mild exhibit poor efficacy in the presence of maternally derived antibodies and against very virulent IBDV⁷. Intermediate and intermediate plus (also called hot) vaccines have a much better efficacy and may break through higher levels of maternally derived antibodies⁷, but can induce moderate to severe bursal lesions causing corresponding levels of immunosuppression^{8,9}. Inactivated IBDV is thought to contain sufficiently high antigenic content to stimulate protective immune response against bursal infection⁷. It is also thought that different IBDV strains induce different levels of humoral immunity¹⁰. Immunogenicity of a vaccine is a prerequisite to protection.

The purpose of this study therefore was to determine whether IBDV strains isolated from field outbreaks in Kenya, and inactivated with formalin were immunogenic in indigenous chickens and therefore suitable for the development of killed vaccines. To investigate this, we measured immune response to formalin killed IBDV isolates obtained from outbreaks that occurred in various parts of Kenya.

Materials and methods

Viruses

Five archived (-20°C) IBDV isolates obtained from five different outbreaks in Kenya were used¹¹. The isolates were designated as E3, E19, E34, E39 and E42 (Table 1).

Isolate E3 was isolated from a flock of broilers in Nakuru where it had caused a mortality rate of 50% (98/200). Isolate E19 was from an outbreak in a commercial layer farm in Kiambu County exhibiting a mortality rate of 25% (50/200), isolate E34 was from a flock of pullets in Nairobi where it had caused a mortality rate of 100% (1154/1154). Isolate E39 from Nakuru had caused 12% (40/328) mortality rate in a flock of pullets. while isolate E42 was from a flock

of indigenous chickens in Kilifi County where 100% (14/14) mortality rate was recorded.

Viruses were isolated through four serial passages in 11-day-old specific antibody negative (SAN) indigenous chicken embryos followed by amplification in 4 week old IBDV antibody negative indigenous chicks as described by Mutinda *et al.*¹¹. Bursae of Fabricius from these birds were collected 72 hours post-inoculation and a 20% bursal derived virus suspension prepared in Phosphate Buffered Saline (PBS) and treated with antibiotics (streptomycin and penicillin at 1000 $\mu\text{g/ml}$ each)^{11,12}. Pathogenicity of the isolates had been characterised in another study. All viruses belonged to serotype 1 - very virulent IBDV pathotype (vvIBDV)¹³.

Table 1: Description of the flocks from which the outbreak isolates were obtained and mortality rates recorded

Isolate	County of origin	Type of flock	Mortality rate
E3	Nakuru	Broilers	50% (98/200)
E19	Kiambu	Layer pullets	25% (50/200)
E34	Nairobi	Layer pullets	100% (1154/1154)
E39	Nakuru	Layer pullets	12% (40/328)
E42	Kilifi	Indigenous chickens	100% (14/14)

Titration of the viruses

Virus isolates were titrated using embryonated eggs¹¹. From the initial 20% viral suspension, 10 fold dilutions serial were made. Five 11 day old indigenous chicken embryos were each inoculated with 0.2ml of each suspension via the chorio-allantoic membrane route. The embryos

were candled twice daily for seven days. Embryo mortality and infectivity (indicated by congestion and/or haemorrhages) were recorded.

Inactivation of viruses

Inactivation of the virus was achieved by adding 10 μl of formalin (40% formaldehyde) to 1990 μl of virus suspension to make 2mls of virus suspension in 0.2% formalin concentration. This mixture was incubated in darkness overnight (24hrs) at 21°C¹⁴. A fresh virus suspension was prepared at each inoculation.

Experimental chickens

Fertile eggs from SAN indigenous chickens were purchased from an isolated farm in Nairobi. The farm had no history of IBD outbreak and chickens in the farm were not vaccinated against IBDV. Chicks hatched from these eggs were raised at the University of Nairobi, Kabete campus for the duration of the study. They were provided with feed and water *ad-libitum*. Before the start of the experiment, sera from these indigenous chickens were confirmed to be free from IBDV antibodies, through agar gel precipitation test (AGPT) and enzyme linked immunosorbent assay (ELISA)¹². In addition, two birds were humanely sacrificed, Bursae of Fabricius harvested and confirmed negative for IBDV prior to vaccinations. At the age of 4 weeks the birds were divided into six groups of six birds each and transferred to inoculation rooms.

Animal vaccination and sampling

Five out of the six experimental groups were each inoculated with 0.3mls (intramuscular via the leg muscle) of 10⁴EID₅₀ inactivated virus. The last group was a control. Water and feeds were provided *ad-libitum*. Birds were inoculated on days 0 and had boosters on days 14 and 21, while bleeding for serum was done on days 0 (baseline), 14, 21, 28 and 35 post first

inoculation. Baseline serum was collected before inoculation. The experiment was terminated on day 35 and all birds were bled, serum harvested and stored for further tests. Harvested serum was assayed for IBDV antibodies using AGPT and ELISA test.

Procedure for Enzyme linked immunosorbent assay

An IBDV Enzyme linked Immunoassay test kit (IDEXX IBD-XR from IDEXX laboratories USA) was used to determine IBDV antibodies in test serum samples according to the manufacturer's instructions. In brief reagents were allowed to attain room temperature (25°C). These reagents were; 1. Diluent buffer preserved with sodium azide 2. Goat anti-chicken horse radish peroxidase conjugate preserved in gentamycin 3. Stop solution 4. IBD negative control (chicken sera non-reactive for anti-IBD preserved in sodium azide) 5. Positive control (Chicken anti-IBD preserved in sodium azide) 6. TMB substrate. Test serum samples collected from experimental birds were also allowed to attain room temperature (25°C). Reagents and samples were mixed by inverting and swirling gently. Recombinant IBDV antigen coated plates were removed from the IDEXX IBD-XR kit, placed on the working bench and sample positions recorded. The plates had 96 wells per plate arranged in 12 columns and 8 rows. The serum samples were diluted five hundred fold (1:500). At this dilution sample absorbance best reflects the sample titre¹⁵.

Sample diluent used was PBS. 100µl of diluted serum was dispensed into labelled wells. Each sample was tested in duplicate. The negative and positive controls were also dispensed in duplicate. After incubating for 30 minutes at room temperature (25°C), the supernatant from each well was aspirated and discarded. Each well was washed 5 times with 350µl of distilled water. 100µl of

goat anti-chicken horse radish peroxidase conjugated detection antibody was added to each well. The plate was then incubated for another 30 minutes at room temperature (25°C), and then washed again as previously described. 100µl of TMB substrate was added into each well and incubated for 15 minutes in a dark cupboard at 25°C. TMB (3,3',5,5'-tetramethylbenzidine), a chromogen, yields a blue colour when oxidized by hydrolysis of hydrogen peroxide. 100µl of stop solution was then dispensed into each well. Absorbance values were measured and recorded at 650nm, A(650).

The relative level of antibody in the sample was determined by calculating the sample to positive (S/P) ratio¹⁶ and the titre calculated relative to an end point titre at 1:500 dilution according to the formula given below:

$$\text{Log}_{10} \text{Titre} = 1.09(\log_{10} \text{S/P}) + 3.36 \quad [15]$$

The technique is significant because of the near linear relationship between the antibody titre and absorbance value at single working dilution¹⁷.

Procedure for Agar gel precipitation test

This test was carried out as described by Okoye and Uzoukwu¹⁸ but with minor modifications. Agar gels were prepared as described in the OIE (2016) manual of standards. A hexagonal pattern of one central and six peripheral rounded wells (6mm in diameter 3mm apart) were cut using a template and tubular cutter¹². The well at the centre was filled with 50µl of standard IBD antigen; while the five peripheral wells were filled with test sample serum alternated with known standard IBD antiserum (Standardized antigen, Cat No. RAA0123 (IBDV Serotype 1 Antigen) and standardized antisera, Cat No. RAB0124 (IBDV Serotype 1 positive serum) from Animal Health and Veterinary Laboratories

Agency, United Kingdom. Results were read by checking for an opaque white line of precipitation between the central well and peripheral wells; this happened where homologous antigens and antibodies met at optimal concentration¹².

Data Analysis

The data was analyzed using IBM SPSS statistics software. The ELISA titers were recorded and analyzed using the means calculated for each vaccine type. Significance of the variations on antibody titer values was determined by the use of analysis of variance (ANOVA). A p value of p=0.05 was considered statistically significant. Data from AGPT results was qualitative and it was tabulated as ratio of number positive against total number tested¹⁹.

Ethical considerations

This study did not engage any human subjects as part of the experiments or any aspect of the study. The study was evaluated, approved and guided by the Faculty of Veterinary Medicine, University of Nairobi Biosafety, Animal Use and Ethics Committee. All applicable guidelines for the care and use of animals were followed as advised by the Committee.

Results

Detection of antibodies by Agar gel precipitation test

Antibodies were detected in all the birds by day 14 post initial inoculation except for 2 out of the 6 birds inoculated with isolate E34 (Table 2). Serum samples from birds inoculated with isolates E3, E19, E39, and E42 yielded strongly visible precipitation lines across all 6 birds inoculated in each group. Of the 4 birds with detectable lines among those inoculated with isolate E34, 2 yielded strongly visible lines and 2 weakly visible lines. There were strong precipitation

lines in all birds at 21 days post initial inoculation, 7 days post 1st booster. These lines persisted up to day 35 when the experiment was terminated; 35 days post initial inoculation, 21 days post 1st booster and 14 days post 2nd booster.

On day 14 all the serum samples yielded titres below 396 (considered positive) except for isolate E34 which yielded a titre of 831.9 (Figure 1 and 2).

Table 2: Agar gel precipitation test results presented as ratio of number positive against number inoculated per isolate per day of bleeding the inoculated chicks

Isolate	Number of birds inoculated	Number positive / number inoculated on days 14, 21, 28, 35			
		Day 14	Day 21	Day 28	Day 35
E3	6	6/6	6/6	6/6	6/6
E9	6	6/6	6/6	6/6	6/6
E19	6	6/6	6/6	6/6	6/6
E34	6	4/6	6/6	6/6	6/6
E39	6	6/6	6/6	6/6	6/6
E42	6	6/6	6/6	6/6	6/6

Detection and quantification of antibodies by enzyme linked immuno-assay test

In addition antibodies against E34 were detectable in all the six birds by day 14 post inoculation, a variation from AGPT results.

All the titres increased progressively from day 14 through day 28 then dipped on day 35 for isolate E3, E19 and E39 while a progressive increase to day 35 was noted in birds inoculated with isolate E42 and E34. The highest antibody titres were recorded in birds inoculated with isolate E19 followed by E3.

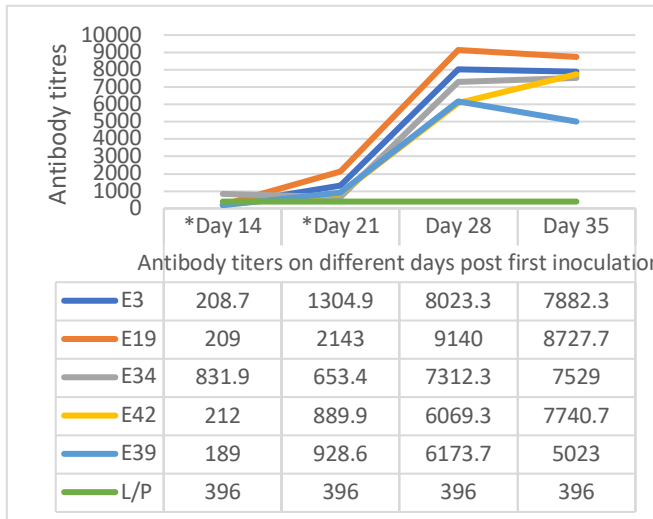


Figure 1: Antibody titres obtained by Enzyme immunoassay test of sera harvested on different days post first inoculation of indigenous chicks with different formalin killed viral isolates.

These two isolates had consistently high titres throughout the experiment. The variations observed between the isolates were not statistically significant ($p=0.9639$). All ELISA titre values on days 21, 28, 28 and 35 were positive. A titre of 396 and above is deemed positive.

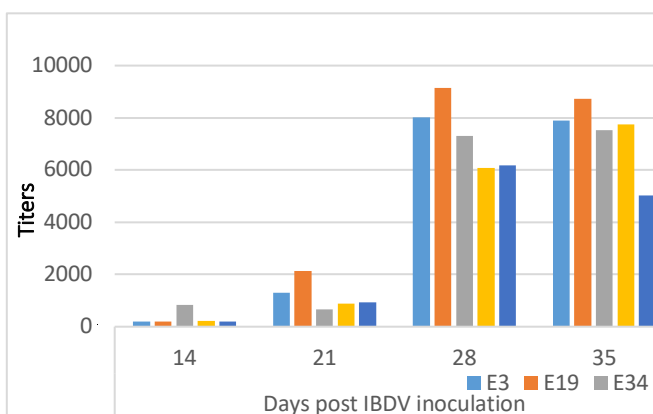


Figure 2: Antibody titres obtained by Enzyme linked immunosorbent assay (ELISA) in indigenous chicken following vaccination with inactivated infectious bursal disease virus (IBDV) isolates administered on days 0, 14, and 21 (Note:

A titre above 396 is deemed protective; all titres were above 396 by day 21).

Discussion

The purpose of this study was to determine whether IBDV strains isolated from field outbreaks, and inactivated with formalin were immunogenic in indigenous chickens in Kenya and therefore may offer potential for the development of killed vaccines. The IBDV isolates in this study were found to be immunogenic. This is in agreement with other studies where bursal derived pathogenic IBDV strains when inactivated and used as vaccines showed high immunogenicity²⁰.

Indigenous chickens are produced under extensive systems with low biosecurity level⁵. This makes vaccination the key method of control of IBD in these chickens. In low biosecurity production systems, killed IBD vaccines can provide protection to young chickens⁷. Further, killed vaccines are suitable as boosters in breeder layers to provide protective maternal antibodies to the progeny.

The four isolates in this study elicited production of high immune responses as detected by both the ELISA and AGPT techniques. The AGPT technique being less expensive and easier to perform¹² compared well with the more expensive ELISA for antibody detection post immunisation although AGPT is less sensitive compared with ELISA^{12,21}. The superior sensitivity of ELISA to AGPT was confirmed in this study as shown on day 14 with isolate E34. This variation was evident when the antibody titres were low as was observed in this study. In a successful vaccination exercise where the titres are presumed to be high, either of the two tests may be used for sero-monitoring¹².

The virus isolates in this study came from field outbreaks making them good possible vaccine candidates due to similarity

in antigenicity with the field strains. Killed vaccines prepared from indigenous strains have been observed to provide better protection due to more antigenic relatedness^{22,23}. Live vaccine viruses multiply in host cells, they are shed and transmitted horizontally. Intermediate and intermediate plus live vaccines which have high efficacy have residual pathogenicity and can cause serious IBD outbreaks in case of reversion to virulence. Live mild vaccines without any residual pathogenicity are less immunogenic⁷. Low immunogenicity of vaccines could result in vaccine failure unless used with highly immunogenic killed vaccines in a prime boost vaccination programme. Farmer vaccinated flocks, vaccinated with imported live IBD vaccines, recorded low immune responses in four farms that were sero-monitored in Kenya²⁴.

Generally, inactivated whole viruses, viral subunits or recombinant viral antigens lack efficient immunogenicity unless they are combined with supporting adjuvants and administered in repeated injections or follow suitable priming with a replicating antigen^{7,25}. In this study immune response with titres as high as protective titres was elicited in non-primed indigenous chickens using formalin killed IBDV local isolates without adjuvants but using repeated boosters. After administering the 2nd and 3rd boosters on days 14 and 21, the titres were much higher than the 396 deemed positive¹⁵ and this agrees with work done by other researchers²⁵. The titres decreased on day 35 confirming the importance of maintaining immunogenicity of killed vaccines either with repeated booster injections or addition of an adjuvant²⁶.

In Nigeria, Angani *et al.*²⁵, while comparing efficacy of indigenous killed and live vaccines in Isa Brown chickens, demonstrated that an immune response elicited after administering a double dose of

IBDV killed vaccine a week apart reached a titre of 893±458 on ELISA and protected the birds against the challenge²⁵. Although immunogenicity does not equal protection, protective antibody titres are elicited by immunogenic vaccine strains.

In spite of vaccinations done with live attenuated vaccines to control the disease, outbreaks of infectious bursal disease still occur in vaccinated flocks⁹. Chicks vaccinated with live IBD vaccines followed by vaccination with inactivated vaccines produce higher immune responses than either live or dead vaccines alone^{7,27}.

All the isolates in this study were immunogenic and could be developed further to be suitable for use in indigenous chickens, a prime boost vaccination programme and breeder layers booster vaccination programme. In particular isolates E19 and E3 could be considered for further development since they consistently yielded very high titres. On the other hand, isolates E34 and E42 may also be considered since they additionally showed potential to elicit an immune response that increased progressively to day 35.

Author contribution

LC and LW conceived the idea, PN and LW designed the experiments, PN, PG, LC and LW assisted in implementation, WU carried out the experiments, PN and WU analysed the data, WU wrote the manuscript, LC, PG, LW and PN corrected drafts of the manuscript.

Declaration of Interest

The authors declare no conflicts of interest.

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