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Production and efficacy testing of live attenuated and inactivated vaccines against experimental *Salmonella* Kentucky infection in broiler chickens

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Abstract:

Background: *Salmonella* Kentucky is of great veterinary and public health concern and exhibits the capacities to emerge as the most prominent *Salmonella* serotype in human disease. The objective of this study is to evaluate the efficacy of prepared live attenuated and inactivated vaccines against experimentally induced salmonellosis caused by *S.* Kentucky in broiler chickens.
Methods: Field isolate of *S.* Kentucky was obtained from the National Veterinary Research Institute Vom, Nigeria and used in the preparation of experimental vaccines. Broiler chickens purchased and ascertained to be free of Salmonellae were randomly distributed into three experimental groups of 20 chicks each. Five-bromouracil at a concentration of 1000µg/ml was used in attenuation of *S.* Kentucky in preparation of the live vaccine and 0.6% formaldehyde was used in inactivation of *S.* Kentucky for preparation of the inactivated vaccine. Group 1 chicks were immunized with the live vaccine subcutaneously at 0.5ml per bird, group 2 received 1ml of the inactivated vaccine subcutaneously, and group 3 served as unvaccinated/control.
Results: There was 100% faecal inhibition and 100% vaccinal efficacy in broiler chickens immunized with the live vaccine on day 56 of age, and 49.6% faecal inhibition and 82.8% vaccinal efficacy in broiler chickens immunized with the inactivated vaccine on day 56 of age when compared with the unvaccinated control. The vaccinated birds also showed higher antibody (IgY) titre levels from day 21 to day 56 of age ($p<0.001$) on all sampling days when values were compared with the unvaccinated birds.
Conclusion: The interplay between vaccination protocol that includes administration of live attenuated or inactivated *S.* Kentucky vaccines and developed antibody (IgY) can reduce intestinal colonization by *S.* Kentucky and subsequent shedding of the serovar in faeces.

Keywords: *Salmonella* Kentucky, 5-bromouracil, IgY, Antibody, Vaccine

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Production et tests d'efficacité de vaccins vivants atténués et inactivés contre une infection expérimentale à Salmonella Kentucky chez des poulets de chair

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Abstrait:

Contexte: *Salmonella* Kentucky est un grand problème vétérinaire et de santé publique et présente les capacités d'émerger comme le sérotype de *Salmonella* le plus important dans les maladies humaines. L'objectif de cette étude est d'évaluer l'efficacité des vaccins vivants atténués et inactivés préparés contre la salmonellose induite expérimentalement par S. Kentucky chez les poulets de chair.
Méthodes: L'isolat au champ de *S.* Kentucky a été obtenu auprès de l'Institut national de recherche vétérinaire de Vom (Nigéria) et utilisé dans la préparation de vaccins expérimentaux. Les poulets de chair achetés et confirmés exempts de salmonelles ont été répartis au hasard en trois groupes expérimentaux de 20 poussins chacun. Le cinq-bromouracile à une concentration de 1000µg/ml a été utilisé pour atténuer *S.* Kentucky dans la préparation du vaccin vivant et 0,6% de formaldéhyde a été utilisé dans l'inactivation de *S.* Kentucky pour la préparation du vaccin inactivé. Les poussins du groupe 1 ont été immunisés avec le vaccin vivant par voie sous-cutanée à 0,5ml par oiseau, le groupe 2 a reçu 1ml du vaccin inactivé par voie sous-cutanée et le groupe 3 a servi de vaccin/vaccin non vacciné.
Résultats: Il y avait 100% d'inhibition fécale et 100% d'efficacité vaccinale chez les poulets de chair immunisés avec le vaccin vivant au 56e jour, et 49,6% d'inhibition fécale et 82,8% d'efficacité vaccinale chez les poulets de chair immunisés avec le vaccin inactivé le 56e jour par rapport au témoin non vacciné. Les oiseaux vaccinés ont également montré des niveaux de titres d'anticorps (IgY) plus élevés du jour 21 au jour 56 (p<0,001) tous les jours d'échantillonnage lorsque les valeurs ont été comparées avec les oiseaux non vaccinés.
Conclusion: L'interaction entre le protocole de vaccination qui comprend l'administration de vaccins *S.* Kentucky vivants atténués ou inactivés et les anticorps développés (IgY) peut réduire la colonisation intestinale par *S.* Kentucky et l'excrétion subséquente du sérovar dans les fèces.

Mots-clés: *Salmonella* Kentucky, 5-bromouracile, IgY, anticorps, vaccin

Introduction:

The global incidence of non-typhoidal *Salmonella* (NTS) gastroenteritis in 2010 was estimated to be 94 million cases, 80.3 million of which were via food-borne transmission, with 155,000 deaths (1,2). Non-typhoidal *Salmonella* can also cause severe extra-intestinal, invasive bacteremia, which is referred to as invasive non-typhoidal salmonellosis (iNTS) (3). The most widely reported serovars associated with iNTS across Africa were *Salmonella* serovar Typhimurium (serogroup B) and *Salmonella* serovar Enteritidis (serogroup D) (4, 5), but over the last decade, there have been a significant shift in the predominant *Salmonella* serovar associated with poultry and human infections (6,7).

Salmonella Kentucky is now widely spread across Africa, and a virulent strain of this serovar with multi-locus sequence type (MLST) ST198 which was reported to originate from Egypt, have been isolated in Canada, Europe and Asia, raising global public health concerns (7,8). This strain is known to be multi drug resistant (MDR) and has accumulated various chromosomal resistance determinants, encoding resistance to multiple antimicrobials including β-lactam antibiotics, carbapenems, quinolones, aminoglycosides, co-trimoxazole (trimethoprim-sulfamethoxazole), and azithromycin (7, 9-12) causing infection in humans probably through consumption of contaminated foods especially poultry meat (12).

Molecular characterization of *S.* Kentucky (MLST sequence type ST198) shows a chromosomal genomic island carrying the resistance genes that confer resistance to these antimicrobials (7), and another matter of concern is the expanding livestock reservoir for this strain. Initially identified in African autochthonous poultry, it has subsequently been found in various animals and food items, and this has allowed for a widespread transmission and propagation of this virulent strain (12-17). In Africa, sources and modes of transmission of NTS are still poorly understood due to the lack of coordinated national epidemiological surveillance systems (18) but poultry flocks have been reported to contribute to its dissemination, as this strain has now been isolated from chickens in Ethiopia, Nigeria and Togo, and from turkeys in Morocco (7).

In Nigeria, there is a high rate of production of poultry and this signifies a high rate consumption of its products; however, this

also means a concomitant increase in the cases of *Salmonella*-associated gastroenteritis (19). This high rate consumption of poultry products can be attributed to its availability and acceptance across several ethnic, religion and diverse cultural backgrounds in the country (20-22). Several reports have documented the spread of *S.* Kentucky serovar across all the geopolitical regions in Nigeria (23-25), and Raufu et al., (26) identified the presence of the virulent *S.* Kentucky MLST type ST198 clone that encode genes resistant to multiple antimicrobials in Nigeria.

A reduction of the burden of *S.* Kentucky associated salmonellosis in humans will involve the elimination of the infection from poultry (27), and this may be achieved through the use of vaccination regimens that will prevent proliferation of the bacteria in poultry. In chickens, vaccines should prevent intestinal and caecal colonization, resulting in diminished faecal shedding and should be effective against systemic infection, preventing vertical transmission and egg contamination (28). A lot of experimental vaccines against salmonellosis have been produced for chickens, and a variety of commercial vaccines are also available in the market (29). Most of these commercial vaccines do not belong to the same serogroup as *S*. Kentucky and thus confers little or no protection against this serotype. In order to reduce the spread of the circulating virulent strain of *S*. Kentucky in birds and its subsequent transmission to man, this study proposes to produce and evaluate the efficacy of a live attenuated and inactivated *S.* Kentucky vaccines in broiler chickens.

Materials and methods:

Study setting

The study was carried out at National Veterinary Research Institute Vom, located in Jos south Local Government Area (LGA) of Plateau State, Nigeria. Experimental vaccines were produced in the Bacterial Vaccine production division and the challenge study was undertaken in the Animal Experimental house of the Institute.

Bacteria isolate

Field isolate of *S.* Kentucky from previous study (24) was obtained from the Bacterial Research Division of the National Veterinary Research Institute (NVRI), Vom, Nigeria, which was identified and serotyped according to the Kauffmann-White Scheme at the OIE *Salmonella* Reference Laboratory, Istituto Zoopofilattico Sperimentale delle Venezie, Padova, Italy.

Experimental birds

A total of 100 apparently healthy day-old White Cornish broiler chicks were obtained from Ibadan (Zartech), Southwest Nigeria. Chicks were randomly distributed into five Pens (20 chicks per pen; two pens for attenuation study and three pens for vaccinal study). Their wings were tagged according to their study group. The procedures used for animal care, housing and experimentation were in accordance with the Ahmadu Bello University (ABU) Committee on Animal Use and Care and ethical clearance from NVRI, Vom, Nigeria.

Culture and isolation of faecal *Salmonella* from the chicks to ascertain their carriage of *Salmonella* spp were done according to the ISO 6579:2002 (30). Cloacal swabs were taken and pre-enriched in buffered peptone water (Oxoid, UK) in a 1:10 sample to broth ratio and incubated at 37°C for 18 hours. 0.1ml of the pre-enriched broth was inoculated into Rappaport-Vassiliadis (RV) (Oxoid, UK) enrichment medium and incubated for 18 hours at 37°C. Samples from the enrichment medium were then plated onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK). The plates were incubated at 37°C for 18 hours, and chicks positive for *Salmonella* spp were excluded (24).

Attenuation of *Salmonella* Kentucky using 5-bromouracil

The methodology of Hertman et al., (31) was adopted with modifications. Stock culture of field isolate of *S.* Kentucky were produced by picking a discrete colony of *S.* Kentucky from XLD agar (Oxoid, UK) plates and inoculating into 10ml Nutrient Broth (NB) (Oxoid, UK), incubated at 37°C for 24 hours. The NB culture containing *S.* Kentucky were further inoculated separately into five NB (Oxoid, UK) (1ml stock culture to 10ml NB) in a graduated 50ml bottle, and incubated at 37°C for 18 hours. The cultures were harvested by centrifugation at 1,000 rpm for 10 minutes. The sediments were washed and re-suspended in 10ml citrate buffer (pH 5.5). One ml each of the culture was taken for colony count (CFU/ml) by adding to 9ml of 0.85% saline (normal saline) in a 10-fold serial dilution in 10 test tubes, with aliquots (0.05ml) from each of the last five dilutions spread onto XLD agar (Oxoid, UK).

Varying amounts of 5-bromouracil (Sigma-Aldrich, Germany) at the following final concentrations in citrate buffer; 400µg/ml,

600µg/ml, 800µg/ml, 1,000µg/ml and 1,200 µg/ml were added to the bacterial suspensions (31), which were incubated in a water bath for 30 minutes at 37°C. After 30 minutes, 0.5ml each of the culture was further taken for viability and colony count by 10-fold serial dilutions of the bacteria in 0.85% saline (normal saline) and subsequent plating of 0.05 ml aliquots on XLD agar (Oxoid, UK).

The treated bacteria culture was then re-centrifuged at 10,000 rpm for 10 minutes and sediment washed twice with 10ml phosphate buffer (pH 7.2). The washed sediment was re-suspended in 10ml fresh NB (Oxoid, UK) and incubated for 18 hours at 37°C in a flask. At the varying concentrations of 5-bromouracil (5-BU), the percentage live bacterial count was determined. Only cultures with less than 20% bacterial live count after exposure to 5-BU were taken for further isolation. Bacterial colonies surviving 5-BU treatment were picked up by sterile Pasteur loops, purified by streaking on XLD agar (Oxoid, United Kingdom) plate surface twice and re-isolation of single colonies following incubation at 37°C for 24 hours (31).

Virulence attenuation test

Infective dose of 5-BU attenuated and non-attenuated *S.* Kentucky stock was prepared to an approximate stock dilution of $1x10^9$ CFU/ml bacteria suspension in sterile NB (Oxoid, UK). Two groups of twenty 14-day-old broiler chicks were infected separately by oral administration by gavage of 1ml of these broth cultures and observed for clinical signs such as profuse white diarrhoea, depression, somnolence and mortality, and gross morphological lesions such as enlargement and necrotic foci in the liver (32,33).

Re-isolation and quantification of *Salmonella* from the liver was done by first homogenizing 1g of liver sample in 10ml of 2% buffered peptone water (Oxoid, UK), and inoculating 1ml of this pre-enriched broth into Rappaport-Vassiliadis (Oxoid, UK), followed by incubation for 18 hours at 37°C. Colony count by 10 fold serial dilution of the bacteria in 0.85% saline (normal saline) was done with subsequent plating of 0.05ml aliquots on XLD agar (Oxoid, UK) plates. The number in CFU/ml transformed to Log_{10} and percentage re-isolation of *S.* Kentucky from liver on XLD agar (Oxoid, UK) was calculated (34,35,36) using the formula; Percentage (%) Re-isolation = $Log_{10}N/9.0x100$, where $Log_{10}N$ = Log_{10} CFU/ml, following Re-isolation, 9.0 = Log_{10} ($1x10^9$ CFU/ml) (represents infective dose and denominator).

Preparation of *Salmonella* Kentucky vaccine stock

Nutrient agar slants were first prepared in Roux flask by suspending 28g of Nutrient agar powder (Oxoid, UK) in 1 litre distilled water. The suspension was brought to boil to dissolve completely, and 250ml dispensed into each Roux flask and sterilized by autoclaving at 121°C for 15 minutes. The Roux flasks were slanted on the bench in sterile room at 25°C to solidify. Aseptically, 10ml of stock cultures of 5-BU attenuated and non-attenuated *S.* Kentucky were inoculated onto the Nutrient agar slant in Roux flasks and incubated at 37°C for 48 hours separately.

The colonies were harvested with sterile normal saline and glass beads by gentle rocking. Purity was carried out on each Roux flask culture by streaking onto Blood agar (BA) plates, and incubating plates at 37°C for 24 hours while Roux flask cultures were stored at 4°C. The 5-BU attenuated and non-attenuated cultures were pooled separately and bacterial suspension adjusted to contain 10^7 CFU/ml using colony count technique (32,36) for vaccine preparation. The purity of the pooled cultures was checked by plating on XLD (Oxoid, UK) and Blood agar plates (32).

Preparation of vaccine stabilizers

Fifteen grams of gelatin (Oxoid, UK) was dissolved in 100ml of deionized water and sterilized by autoclaving at 121°C for 15 minutes. 50g Lactalbumin hydrolysate (LAH) (Oxoid, UK), 100g sucrose (Sigma-Aldrich, Germany), 1.16g potassium dihydrogen orthophosphate (KH_2PO_4) (J.T. Baker, USA) and 7.66g sodium phosphate dibasic (Na_2HPO_4) (J.T. Baker, USA) were dissolved into 1 litre of deionized water and autoclaved at 121°C for 15 minutes. Normal saline was prepared by dissolving 8.5g in 1 litre of distilled water and autoclaved at 121°C for 15 minutes. Purity check was undertaken after 24 hours of incubating all preparations by streaking onto BA plates and incubating at 37°C for 24 hours (32).

Preparation of live attenuated vaccine

Aseptically, the 5-BU attenuated *S.* Kentucky concentrate at approximately 10^7 CFU/ml bacterial suspension was thoroughly mixed with stabilizers at a ratio of 5:4:1 (50% *S*. Kentucky concentrate: 40% LAH: 10% Gelatin). The vaccine was dispensed into sterile vials at 2.5ml per vial and lyophilized at National Veterinary Research Institute, Vom where production of the experimental vaccine was undertaken (32).

Preparation of inactivated vaccine

The non-attenuated *S.* Kentucky concentrate (3 litres) at approximately 10^7 CFU/ml bacterial suspensions was dispensed into sterile 10 litres round bottom flask, 18ml of 37% formaldehyde stabilized solution (Sigma-Aldrich, Germany) was added and thoroughly mixed before plating on Blood agar plate and incubating at 37°C for 24 hours, to ascertain inactivation. Following confirmation of bacterial inactivation, Glycerin saponin saline (GSS) [which has been prepared by mixing 1000ml of glycerin (Schutz, Indonesia) with 100g of saponin (Sigma-Aldrich, Germany) and 8.5g of NaCl (Oxoid, UK) in 1000ml of deionized water and autoclaved at 121°C for 15 mins in graduated 5000ml Schott-Duran bottle] was added to the killed bacteria suspension at a ratio of 8:2 (8 parts of inactivated bacteria suspension: 2 parts of GSS) and the vaccine was dispensed into sterile 200ml bottle (32).

Purity test on live attenuated and inactivated vaccines

Five vials of the lyophilized live attenuated vaccine were sampled randomly and reconstituted with 2.5ml sterile normal saline. Using a Pasteur wire loop, purity check was carried out by streaking each vial separately on paired BA and Sabouraud's Dextrose agar (SDA) plates. BA plates were incubated aerobically at 37°C for 24 hours and anaerobically in jar using CO_2 gas packs (CO_2Gen Oxoid, UK) at 37°C for 24 hours while the SDA plates were incubated at 25°C for 7 days. Colony count was undertaken by a 10-fold serial dilution in normal saline and aliquot (0.05ml) of the last 5 dilutions were cultured on BA plates in duplicate pairs, and incubated at 37°C for 24 hours.

Sterility (inactivation) check on the inactivated vaccine was carried out by streaking a Pasteur wire loop full on BA plate of the prepared vaccine, incubated at 37°C for 24 hours and Gram stain undertaken for the inactivated vaccine (32).

Experimental design

Three groups, each with 20 day-old broiler chicks, were reared in a deep litter system in separate pens; group 1 received 0.5ml of the prepared live attenuated *S*. Kentucky vaccine subcutaneously at day 11 of age and a repeat at day 25 of age; group 2 received 1.0ml of the prepared inactivated *S*. Kentucky vaccine subcutaneously at day 11 of age and a repeat at day 25 of age; group 3 served as the control (unvaccinated) and were inoculated with 0.5ml sterile normal saline subcutaneously at day 11 of age and a repeat at day 25 of age (32).

The three groups were challenged on day 32 of age by oral gavage of 1ml each of *S*. Kentucky field isolate in NB (Oxoid, UK) containing approximately 5 x 10^7 CFU/ml bacteria suspension (34). This was prepared by first suspending six and half grams of NB powder in 500ml distilled water, mixing and dispensing into Schott Duran bottle, and sterilized by autoclaving at 121°C for 15 minutes. Discrete colonies of *S.* Kentucky on XLD agar were then inoculated onto NB and incubated at 37°C for 24 hours. Purity of the culture was checked by plating on XLD agar (Oxoid, UK) and Blood agar plates (32) and colony count was titred to bring to an approximate stock dilution of 5 x 10^7 CFU/ml bacteria suspension (36).

Assessment of vaccine potency

For the assessment of vaccine potency, serum samples of experimental birds were collected on day 7, day 14, day 21, day 28, day 35, day 42, day 49 and day 56 of age and stored at -20°C. Seroconversion of the experimental broiler chickens to *S*. Kentucky was measured in the serum using Chicken IgY Enzyme Linked Immunosorbent Assay (ELISA) kit (Wuhan Fine Biotech Co., Ltd) according to the manufacturer's directive. One µL of serum sample was diluted in 99µl of Standard Dilution Buffer and quantification of titre was done by measuring absorbance at 450 nm wavelength in an ELISA machine (RT-2100C; Rayto Microplate Reader). The ELISA was run at one time for all the serum samples to minimize variability.

Faecal shedding

Quantitative *Salmonella* shed in faeces at baseline and following experimental vaccine administrations were determined by taking cloacal swabs from vaccinated and non-vaccinated birds at day 7, day 14, day 21, day 28 and day 31 before challenge with field *S.* Kentucky isolate. Cloacal samples were then taken on days 35, 42, 49 and 56 after the challenge. Sample swabs were used to collect multiple faecal samples repeatedly from the broiler chickens to a stomacher bag, and 1g of the faecal sample was then diluted in 10ml of 0.85% saline (normal saline). Further, 10-fold serial dilution of the sample in normal saline in 10 test tubes was carried out and 0.05ml aliquots of each of the first five dilutions were spread onto XLD agar plates (Oxoid, UK). After 18 to 24 hours of incubation at 37°C, *Salmonella* colonies (black) on each plate were

counted (35). The prevalence of infection and percentage vaccinal efficacy were calculated using the method of Thrusfield 2007 (38) with the formula; Percentage (%) vaccinal efficacy = C-T/Cx100, where C=prevalence of infection in unvaccinated animals (unvaccinated animals are defined as 'exposed' to the risk factor), and T = prevalence of infection in vaccinated animals.

Data analysis

All data obtained from the study were entered and analysed with the aid of GraphPad Prism (Version 5.01, Graphpad software Inc.). P value < 0.05 was considered statistically significant. The data for the mean quantitative CFU/ml faecal Salmonellae shed and the mean quantitative serum antibody (IgY) titre for the vaccinated groups versus the control/ unvaccinated group over time was analysed using a two-way analysis of variance (ANOVA).

The mean antibody (IgY) titres between the live vaccine group and inactivated vaccine group were compared using paired sample t-Test before and after immunization with the prepared vaccines. The correlation between the mean quantitative serum antibody IgY (Log_{10} ng/mL) titre and the mean quantitative faecal *Salmonella* (Log_{10}CFU/ml) shed was determined by Pearson correlation test (r^2 value). Results were presented in tables, figures and charts.

Results:

Results of virulence test

Profuse white diarrhoea, depression, hurdling, anorexia and somnolence were observed in broilers challenged with the non-attenuated stock culture. There was also depression, anorexia, somnolence and hurdling observed in broiler chickens challenged with the 5-BU attenuated stock, however white diarrhoea was absent as faeces were pasty brown.

Post mortem observable changes three days after challenge was a slightly enlarged and congested liver in broiler chickens challenged with the non-attenuated stock, with percentage hepatic re-isolation of *S.* Kentucky of 62.2%. In broiler chickens challenged with 5-BU attenuated stock, post mortem examination revealed a normal liver and a percentage hepatic re-isolation of 30%.

Vaccinal efficacy against *Salmonella* Kentucky experimental infection

Infection rates in the unvaccinated (exposed) birds on sampling days post-challenge were consistently higher than in the vaccinated birds and this was statistically significant ($p<0.05$), however between the vaccinated groups, there was no statistically significant difference.

On day 35 of age of birds, i.e. 3 days post challenge, 53.9% of birds were protected from infection by the live vaccine and 34.4% of birds were protected from infection by the inactivated vaccine. Vaccinal efficacy increased to 100% in birds immunized with live vaccine and 82.8% in birds immunized with the inactivated vaccine on day 56 (Table 1).

Table 1: Prevalence of infection and Percentage (%) vaccinal efficacy against *Salmonella* Kentucky in broiler chickens after challenge

Age of birds in days/prevalence of infection (percentage vaccinal efficacy)				
Group	Day 35	Day 42	Day 49	Day 56
Live vaccine*	6/18 (53.86)	6/18 (60.02)	5/18 (68.73)	0/18 (100)
Inactivated vaccine*	9/19 (34.35)	7/19 (36.85)	6/19 (64.45)	2/19 (82.82)
Unvaccinated (control)	13/18	15/18	16/18	11/18

*No statistically significant difference ($p>0.05$) between the vaccinated groups

Mean quantitative faecal Salmonellae shed on sampling days post challenge

On all sampling days prior to challenge, faecal *Salmonella* shedding was unnoticed in cloacal swab buttressing apparent safety of the experimental vaccines but on days 35, 42, 49 and 56 of age, there were statistically significant differences (p<0.001) when the vaccinated groups were compared against each other and against the control. Faecal *Salmonella* counts in cloacal swabs of birds immunized with experimental vaccines on day 56 of age were lower than the unvaccinated group, with 0 CFU/ml for the live attenuated vaccine, 3.5 x 10^2 CFU/ml for the inactivated vaccine, and 1.33 x 10^5 CFU/ml for the control group (p<0.05). This represented 100% inhibition by the live vaccine and 49.6% inhibition by the inactivated vaccine when compared to the control (Table 2).

Antibody (IgY) titre in serum

The antibody (IgY) titre of vaccinated birds was significantly higher than those of the unvaccinated birds post vaccination (p<0.001). There was no significant difference in IgY titre levels between the two vaccinated groups on sampling days post vaccination except on day 28 of age post-booster vaccination, when IgY titre in the live vaccine group was significantly higher than the inactivated vaccine group (p<0.05) (Table 3).

Table 2: Mean number of *Salmonella* Kentucky count in CFU/ml re-isolated in faeces after challenge

Age of birds in days/number of CFU per ml				
Group	Day 35	Day 42	Day 49	Day 56
Live vaccine	8.80 x 10^3	1.28 x 10^3	3.50 x 10^2	0
Inactivated vaccine	9.00 x 10^3	2.43 x 10^3	4.75 x 10^2	3.50 x 10^2
Unvaccinated (control)	4.33 x 10^5	2.24 x 10^6	1.34 x 10^7	1.33 x 10^5

p<0.001 when vaccinated groups were compared to control

Table 3: ELISA result showing the mean number of antibody (IgY) titre (ng/ml) in serum of experimental birds

Age of birds in days/antibody titre								
Group	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Live vaccine	2.71	4.44	10.51*	19.85*a	22.15*	23.10*	22.02*	20.56*
Inactivated vaccine	2.44	4.46	11.47*	18.23*	20.81*	22.15*	20.70*	19.12*
Unvaccinated (control)	2.14	3.85	3.06	3.61	8.39	11.51	8.57	8.64

Serum dilution (1:100) thus actual value in 'ng/ml' is multiplying figure in each cell by 100
* = p<0.001 when vaccinated groups values was compared to the unvaccinated group
a = p<0.05 when live vaccine group was compared to inactivated vaccine group
Paired sample t-Test results between live vaccine group and killed vaccine group; p value=0.05
Correlation coefficient between Live vaccine and Inactivated vaccine groups = 0.997

Correlation of antibody (IgY) titre to faecal *Salmonella* shed in vaccinated chickens

The correlation coefficient R^2 for the mean serum antibody (IgY) titre to faecal *Salmonella* shed in broiler chickens immunized with the live attenuated vaccine was 0.8519. This represents a strong positive correlation (Fig 1). At day 35 and 42 of age, antibody titre rose while faecal *Salmonella* shed declined. At day 49 and day 56 of age, there was commensurate decline in serum antibody (IgY) titre and faecal *Salmonella* shed (Fig 1).

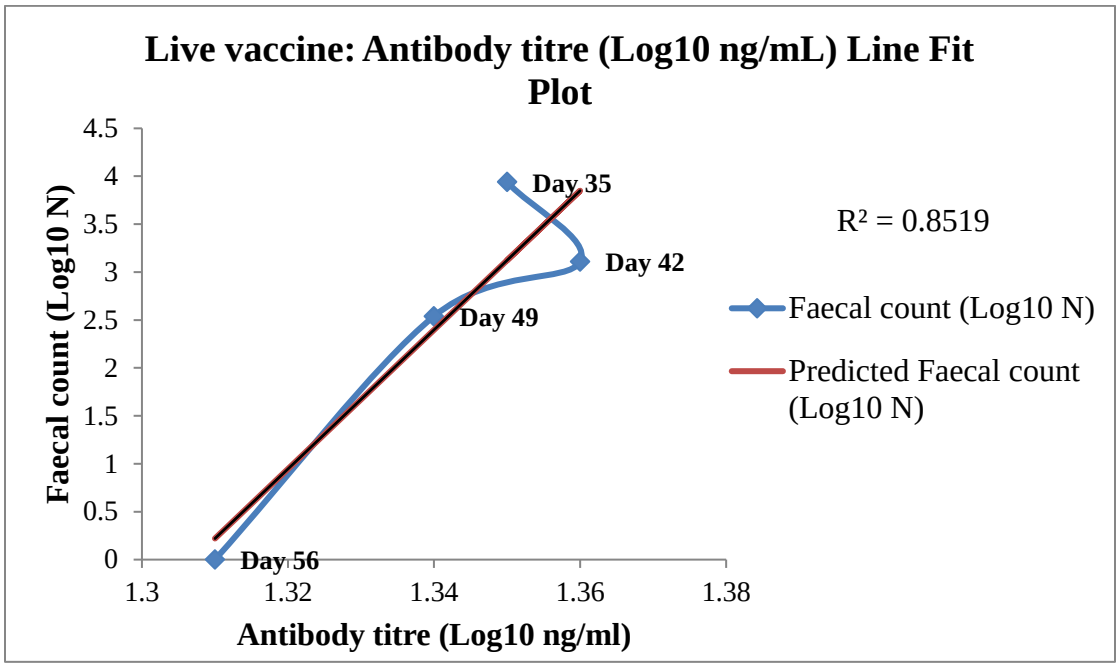

Fig 1: Correlation of the effect of mean Antibody (IgY) titre on the mean faecal Salmonellae shed in broiler chickens immunized with live vaccine; R^2 (0.8519): The correlation coefficient represents a strong positive relationship; *p* value = 0.333

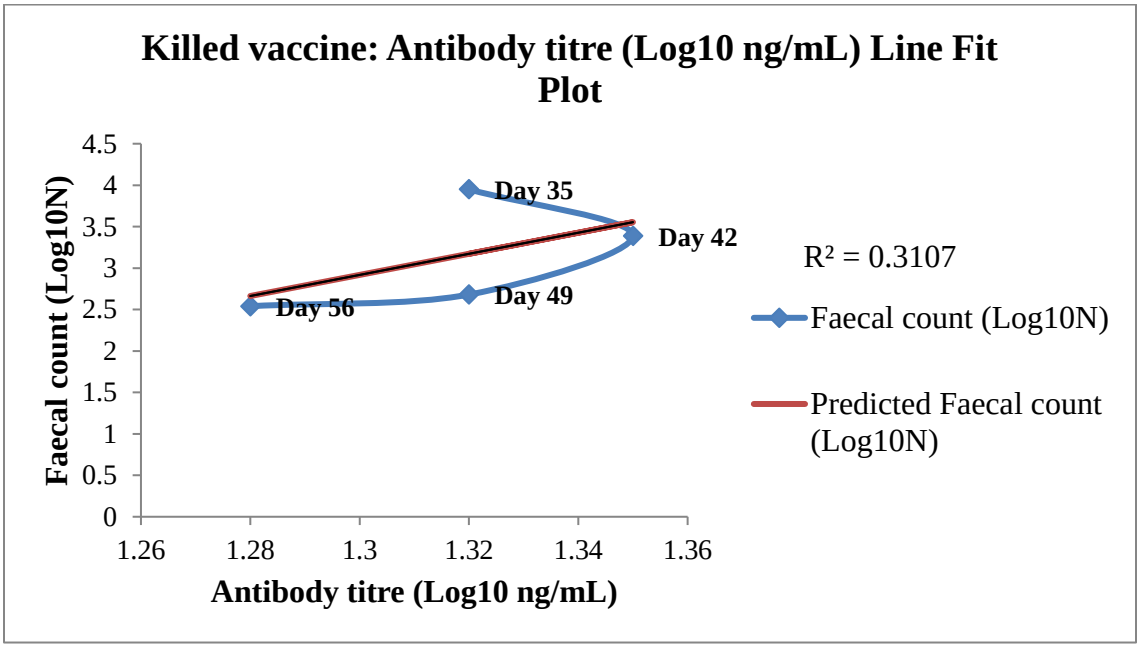

Fig 2: Correlation of the effect of mean Antibody (IgY) titre on the mean faecal Salmonellae shed in broiler chickens immunized with inactivated vaccine; R^2 (0.3107): The correlation coefficient represents weak positive relationship; *p* value = 0.417

The correlation coefficient R^2 for the mean serum antibody (IgY) titre to faecal *Salmonella* shed in broiler chickens immunized with the inactivated vaccine was 0.3107. This represents a weak positive correlation (Fig 2). All sampling days post challenge (day 35, 42, 49 and 56 of age) were not commensurate. At day 35 and 42 of age, the mean serum antibody (IgY) titre rose while faecal *Salmonella* shed declined but on day 49 and 56 of age, serum antibody (IgY) titre and faecal *Salmonella* shed declined (Fig 2).

Discussion:

The live attenuated and inactivated experimental vaccines prepared in this study significantly reduced faecal *S.* Kentucky shed in broiler chickens immunized, when compared to the unvaccinated broiler chickens. This finding is similar to those observed by Clifton-Hadley et al., (39), Papezova et al., (40), Nourhan et al., (41) and Groves et al., (42), who reported that vaccine preparations containing either live attenuated or inactivated *Salmonella* spp. can reduce faecal shed of the bacteria. It has been observed that vaccine preparations containing live organisms in comparison to inactivated ones provide a superior protection in preventing intestinal colonization and environmental contamination by *Salmonella* spp., evident by a higher decline in faecal *Salmonella* shed (39, 40, 43, 44).

In the present study, faecal shedding of Salmonellae between the vaccinated groups did not differ significantly from day 35 to day 42 of age post challenge, but there was statistically significant difference on day 56 of age with broiler chickens immunized with the live attenuated vaccine observed to have superior protection at this day. This superior protection has been attributed to the capacity of live attenuated *Salmonella* vaccines to stimulate the secretion of mediators (cytokines, interleukins and interferons) and inducing a T helper 1 (Th1) response cell-mediated immunity, which does not occur with inactivated products. Additionally, CD8+ T cells play a crucial role in immune protection against *Salmonella*, and these cells (CD8+ T) response are exclusive after vaccination using live attenuated vaccines (45- 48).

The result of the mean serum antibody (IgY) titre in the present study showed that the immunized broiler chickens produced and sustained a superior IgY titre from day 21 of age following first immunization to day 56 of age when compared to the unvaccinated broilers. These findings were similar to those reported by Babu et al., (49) and Tizard (50). The superior seroconversion in immunized broiler chickens was attributed to the effect of the prepared vaccines. On day 42 of age, antibody (IgY) titre in serum peaked in all the groups, reaching 2310 ng/mL in broiler chickens immunized with the live vaccine preparation, 2215 ng/mL in the inactivated vaccine group and 1151 ng/mL in the unvaccinated group. There was also a rapid rise in antibody (IgY) titre in the broiler chickens on day 35 of age across all groups, which may be related to an increase mobilization of antibody (IgY) due to the introduction of live bacteria, similar to what was observed by Nourhan et al., (41).

A strong positive correlation of 0.997 in antibody (IgY) titre between the vaccinated groups was observed in this study and mean paring was significantly effective (p=0.05). This finding showed that neither the live attenuated preparations nor inactivated preparation was superior in eliciting serum antibody (IgY) titre, as immune response judge by antibody (IgY) titre produced were closely matched throughout the sampling days. This was in contrast to reports by Penha Filho and Berchieri (51) who reported a higher level of immunoglobulin levels in birds immunized with an inactivated vaccine than a live vaccine preparation. Papezova et al., (40) also reported a higher immunoglobulin titre in birds vaccinated with inactivated preparation than a live preparation. The result of our study however is similar to Babu et al., (49) who reported that live vaccines have been shown to be more effective in increasing lymphocyte proliferation in response to *Salmonella* antigens in laying hens. Several factors however may cause these variations in humoral immune response elicited in birds (52), as the level of protection depends on the challenge strain/components of the vaccine, route of administration, infection dose, age of birds and species/line of birds. Consequently, it has been difficult to compare strictly the efficacy of the vaccine preparations currently available (52,53). Papezova et al., (40) pre- pared an inactivated vaccine enriched by SPI-1 or SPI-2 proteins, which are proteins central to *Salmonella* virulence that may be responsible for the marked difference.

This present study made use of saponin as an adjuvant, which has been shown to have great characteristic as an immuno-adjuvant by Barbosa (54) while other studies made use of aluminium gels and salts as adjuvant. Adjuvants are defined as groups of structurally heterogeneous compounds that enhance or modulate the immunogenicity of

the associated antigens. Despite the recognition of many different types of adjuvants, however, little is known about their mode of action. The events triggered by these immunomodulators appear to come from one or the combination of several of the following effects; depot effect, effect on antigen presenting cells (APC), nonspecific immuno-stimulating effect and particulated carrier systems (53). There are few and isolated information that explains how vaccines work and the immune response depending on the antigen and route of vaccination (44). Overall, in our study, immunized birds produced and sustained high serum antibody (IgY) levels that were able to provide protection against challenge by *S.* Kentucky proven by the significant decline in the cumulative amount of Salmonellae shed in faeces of immunized birds when compared to the unvaccinated birds.

Conclusion:

Our study demonstrated the interplay between vaccination regimens which include immunization with either 5-BU attenuated live *S.* Kentucky vaccine or 0.6% formalin inactivated *S.* Kentucky vaccine, and the developed antibody (IgY) titre reduced intestinal colonization and interfered with the pattern and shedding of *S.* Kentucky in faeces of broiler chickens. There was 100% vaccinal efficacy and inhibition of faecal shedding of *S.* Kentucky by the live attenuated vaccine as well as 82.8% vaccinal efficacy and 49.6% inhibition of faecal shedding of *S*. Kentucky by the inactivated vaccine preparation in broiler chickens used for the experiment.

Conflict of interest:

Authors declared no conflict of interest

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