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Experimental reproduction of rotavirus and *Salmonella pullorum* gastroenteritis in broiler chicks

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Abstract

Numerous viruses, including astroviruses, reoviruses, rotaviruses, coronaviruses, and adenoviruses, have been implicated as causative agents of enteric disease. This is because they have been isolated from or identified in the intestines and intestinal contents of affected poultry flocks. Four experiments were conducted to reproduce enteritis in broiler birds using rotavirus and *Salmonella pullorum*. Fifty-two broiler birds were obtained and randomly divided into 4 groups. Group A chicks were inoculated with 1 X 10^6 pfu/ml of rotavirus, group B chicks were inoculated with 1 X 10^6 cfu/ml of *Salmonella pullorum*, group C chicks were inoculated with 1 X 10^6 pfu/ml of rotavirus and 1 X 10^6 cfu/ml of *Salmonella pullorum*, while group D birds were given 1ml of PBS alone. Birds in all groups were observed daily for clinical signs and the intestines were processed for histopathological evaluation. Diarrhea and depression were the major signs in chicks given any one of the inoculum. Histological changes were characterized by swollen villus tips and constricted villus bases, proliferation of enterocytes and necrotic villi. Significant growth retardation was observed in chicks given either rotavirus or *Salmonella pullorum*, but this effect was more pronounced in chicks given the combination of rotavirus and *Salmonella pullorum*. The present study reveals that oral inoculation of birds with rotavirus or rotavirus/*Salmonella pullorum* combination leads to diarrhoea and significant growth depression. It is thus important to continuously screen and prevent against organisms infecting the GIT for optimal performance of birds.

Keywords: Broiler, Diarrhea, Growth depression, Rotavirus, *Salmonella pullorum*
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Introduction

Various viruses of unknown importance have been identified in poultry by electron microscopic examination of faeces and intestinal contents (Hazelton & Gelderblom, 2003). Many of these viruses have been identified as the aetiologic agent of gastrointestinal tract infections in chickens. One of such important virus is rotavirus. The virus belongs to the genus Rotavirus and family Reoviridae. It gains entry to the body through oral ingestion, and transmission occurs via faecal shedding. Rotavirus replication occurs primarily in mature epithelial villi of the small intestine (Ruiz *et al*., 2009), and maximum excretion of the virus in faeces occurs from 2 to 5 days post exposure. Diarrhoea is the principal manifestation of the disease in clinically affected birds; decreased weight gain, dehydration and increased mortality also may be observed.

Rotavirus induced diarrhoea is thought to be caused by a combination of factors (Omore *et al*., 2016),

which include a reduction in epithelial surface area, replacement of mature enterocytes, an osmotic effect, secretion of intestinal fluid and electrolytes (Lundgren *et al.*, 2000), and the effect of the rotavirus non-structural protein 4 (NSP4), which acts as a viral enterotoxin (Didsbury *et al*., 2011). Malabsorption and maldigestion has been the major cause of diarrhea in infected birds (Didsbury *et al*., 2011). Rotavirus infections of avian species vary from subclinical to severe infection. This variation in severity of rotavirus infections may be due to differences in virulence among strains or the interaction of other infectious, environmental or management factors (Desselberger, 2014). There have been reports of rotavirus infection in combination with other viruses or bacteria in poultry birds (Pantin-Jackwood *et al.*, 2008; Jindal *et al*., 2009; Mettifogo *et al*., 2014) causing a disease complex. The major enteric disease complex in turkeys is poult enteritis complex (PEC) (Barnes *et al.*, 2000), also known as poult enteritis mortality syndrome (PEMS) in its more severe presentation (Barne & Guy, 2003). Runting and stunting syndrome (RSS), also known as malabsorption syndrome, is the major enteric disease complex in broiler chickens. Since 2004, the broiler industry in the southeastern United States, and more recently in other regions of the world (Day *et al*., 2015), has experienced severe outbreaks of RSS. Many different agents have been detected in these cases including viruses (rotavirus, enterovirus, reovirus, adenovirus), bacteria (*Salmonella* sp., *Escherichia coli*, *Enterococcus*), and protozoa (*Eimeria* sp). Of all the pathogens detected, the proportion of rotavirus and *Salmonella* was greater than any other agent (Jindal *et al*., 2010), and there are no reports of such mixed infection in Nigerian poultry except for rotavirus alone (Oni *et al*., 2017). Therefore, this study was designed to determine clinical signs associated with rotavirus infection alone and its combination with *Salmonella pullorum*, to determine changes in body weight of chicks infected with Rotavirus, Salmonella or the combination of the two organisms and to evaluate the intestinal gross and histopathological changes associated with the disease in broiler chicks.

Materials and Methods

Experimental design

Fifty-two (52) one day old non-diarrhoeic broiler chicks were procured from a local commercial hatchery and were maintained in the experimental unit of the College of Veterinary Medicine, Federal University of Agriculture Abeokuta, Ogun state. Both feed and water were provided *ad libitum*. All the birds were weighed individually at day one to obtain baseline data on body weight (BW) and randomly divided into 4 groups at day 5. Each of the groups (groups A, B, C, and D) contained 13 chicks each. All the groups A, B, C, and D were inoculated via the crop. Group A chicks were inoculated with 1 X 10^6 pfu/ml of rotavirus, group B chicks were inoculated with 1 X 10^6 cfu/ml of *Salmonella pullorum*, group C chicks were inoculated with 1 X 10^6 pfu/ml of rotavirus and 1 X 10^6 cfu/ mlof *Salmonella pullorum*, while group D birds were given 1ml of PBS alone (negative control). The birds were monitored daily till the end of the experiment and observations were recorded twice daily. The virus and bacteria were obtained from Friedrich Loeffler Institut, Germany and Department of Veterinary Microbiology and Parasitology, Federal University of Agriculture Abeokuta, Ogun state respectively.

Ethical consideration

This study was approved by COLVET research ethics committee, Federal University of Agriculture Abeokuta with approval certificate reference number FUNAAB/COLVET/CREC/007/17.

Clinical findings and growth response

All groups were observed daily for the development of clinical signs and mortality. In order to determine the effect of different treatments on BW, chicks from each group were weighed on a weekly basis post infection. Growth depression due to infection was calculated in each experiment by dividing mean BW of chicks in treated groups by mean BW of those in the control group at the last day of the experiment.

Diarrhoea score

The severity of diarrhoeal illness was assessed by examination of faecal material. Diarrhoea was scored from absent (-) to severe (+++) based on color, texture and amount of stool. Normal faeces were scored as absent (-), exceptionally loose faeces was scored as mild (+), loose brownish faeces were scored as moderate (++), and watery faeces were defined as severe (+++). Faeces scored from moderate to severe were considered as diarrhoeic.

Gross and histopathological evaluations

Post mortem examination of chicks that died was performed; live birds were euthanized for gross and histological evaluations. Gross changes of intestinal content and mucosa were observed. Intestine was collected and fixed in neutral buffered 10% formalin

for at least 24 hours. Transverse sections of intestine were cut, dehydrated in alcohol and embedded in paraffin wax. Four sections were placed into each embedding cassette, and cassettes were labeled in ascending order. Paraffin sections were stained with hematoxylin and eosin and examined under an Olympus® light microscope.

Statistical analysis

Mean values (± standard deviation) of weight were calculated on a weekly basis for each group. The differences in mean values was compared by one-way ANOVA at $P<0.05$ probability level using GraphPad Prism 6.

Results

Clinical findings

In general, inoculated chicks in groups A, B and C showed diarrhoea from day 1 P.I. Initially, faeces were watery in consistency and subsequently brownish and watery. A summary of diarrheic faeces observed is depicted in Table 1. More watery faeces for a longer duration were noticed in group C as compared with groups A and B. More mortality was observed in group C with some birds showing signs of dullness and depression. Dullness and depression were also evident in a few chicks in all treated groups (except the control group) at different days of observations. Lack of uniformity was evident in all treated groups from day 7 P.I. Group A, B and C chicks exhibited watery faeces between day 1 and 15 P.I. None of the group D chicks (control group) exhibited depression, lethargy or died during the experiment.

Body weight

Growth retardation was observed from day 7 P.I. in all infected group till the end of the study. The effects of Rotavirus and Salmonella organism in broiler chicks are depicted in Table 2. Body weights

Table 1: Diarrhoea score in birds inoculated orally with rotavirus, Salmonella or rotavirus/Salmonella combination.

	Group diarrhoea score			
Age (DPI)	A (Rotavirus)	B (*S. pullorum*)	C (Rotavirus/*S. pullorum*)	D (PBS)
1	+	+	+	-
2	++	+	++	-
3	++	++	+++	-
4	++	++	+++	-
5	++	++	+++	-
6	+++	+++	+++	-
7	+++	+++	+++	-

DPI: Days post infection

Note: - = absent, + = mild, ++ = moderate, +++ = severe

Table 2: Mean body weights of birds inoculated orally with Rotavirus, Salmonella or Rotavirus/Salmonella combination

Days post infection (DPI)	A (Rotavirus)	B (*S. pullorum*)	C (Rotavirus/ *S. pullorum*)	D (PBS)
7	144±22.1^{a}(4)	147.6±14.4^{a}(2)	140.0±30.9^{a}(7)	150.6±15.8^{a}
14	245.9±25.7^{a}(16)	244.0±30.6^{a}(16)	215.5±54.3^{a}(26)	292.2±35.8^{b}
21	342.1±5.0^{a}(15)	348.1±45.1^{a}(14)	305.3±52.2^{a}(24)	403.9±26.8^{b}
28	428.5±22.9^{a}(20)	435.8±23.5^{a}(18)	415.8±29.14^{a}(22)	534.4±21.1^{b}
35	509.0±9.4^{a}(20)	511.8±8.4^{a}(19)	508.8±7.1^{a}(20)	634.5±10.9^{b}
42	620.6±12.4^{ab}(25)	631.1±8.6^{a}(23)	606.9±12.2^{b}(26)	824.5±11.4^{c}

Weight in grams Mean± SD at DPI (percentage loss in weight)

Note: A= chicks inoculated with rotavirus alone; B =chicks inoculated with Salmonella pullorum alone; C = chicks inoculated with rotavirus and Salmonella pullorum; D =chicks inoculated with phosphate buffered saline (controls).

All values are mean +/-SD of birds at each interval. Values with different superscripts (a, b, and c) within rows differ significantly ($P \leq 0.05$)

In birds of groups A, B and C were significantly lower than those of birds in group D from day 7 P.I. Among the 4 treatments, birds inoculated with rotavirus and *Salmonella pullorum* (group C) had the lowest BW followed by birds inoculated with rotavirus alone (group A) and *Salmonella pullorum* alone (group B). In groups C, inoculation with rotavirus and *Salmonella pullorum* significantly ($P \leq 0.05$) retarded their growth when compared with group B birds at the end of the experiment. The control group D had a higher body weight than all the other groups. This steadily increased from day 7 P.I till the end of the study.

Gross pathology

At necropsy, no gross changes were observed in chicks of group D (control group). The same gross changes were observed in all the infected birds. These changes were mostly confined to the intestinal tract. Birds in groups A, B and C also had pasted vent. Pale distended intestines with watery contents and distended ceca with loose to watery contents were observed. Gross changes were more severe in group C than in groups A and B and correlated with the duration of diarrhoea in treated chicks.

Histopathology

Histopathological changes in the small intestine of rotavirus infected birds were characterized by swollen villus tips and constricted villus bases (Plate I). In many villi, lesions seemed to be present at the tips. In rotavirus infected birds at 7 DPI, nuclei were enlarged and irregularly positioned within the cells. There was goblet cell hyperplasia with focal areas of necrosis and exfoliated enterocytes. Proliferation of enterocytes with goblet cell hyperplasia and matted villi were observed in the Salmonella-infected group (Plate II). Severe lymphocytic infiltration and necrosis of villi and syncytial formation in the

Table 3: Summary of histopathologic changes observed in the intestine of inoculated birds

Histological changes	A (Rotavirus)	B (*S. pullorum*)	C (Rotavirus/*S. pullorum*)	D (PBS)
Goblet cell hyperplasia	++	++	+++	+
Necrotic changes	++	+++	+++	-
Matted villi with exfoliation of enterocytes	++	+++	+++	-
Proliferation of enterocytes	++	+	+	+
Lymphocytic infiltration	+	++	++	-
Syncycial giant cell formation of the enterocytes	+	+	++	-

Note: - = absent, + = mild, ++ = moderate, +++ = severe

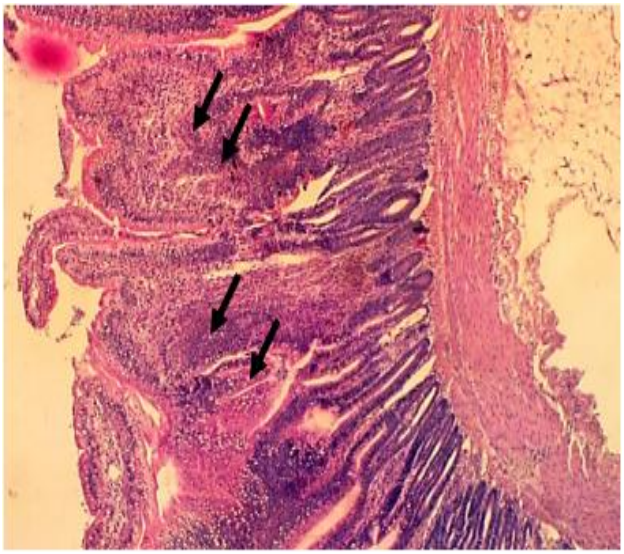

Plate I: : Photomicrograph of intestine of group A bird infected with rotavirus showing lamina propria infiltration by mononuclear cells (arrow) and goblet cell hyperplasia (H&E stain) at 7dpi. x 300

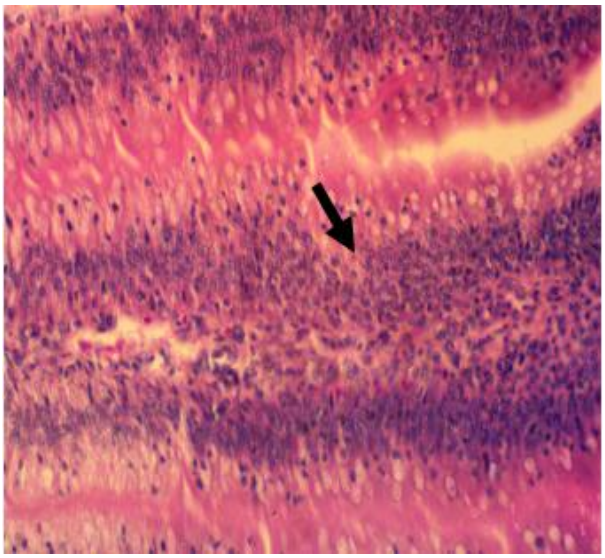

Plate II: Photomicrograph of intestine of group B bird infected with *Salmonella pullorum* showing proliferation of enterocytes (arrows) with marked goblet cell hyperplasia (H&E stain) at 7dpi. x 350

rotavirus and *Salmonella pullorum* infected group (Plate III), some birds also showed severe enterocyte proliferation. At one week interval all cells in the intestine were dead; crypts with no villi were also seen in birds.

Discussion

This study was undertaken to determine signs associated with infection of broilers with rotavirus and *Salmonella pullorum*. After oral inoculation, diarrhoea was the major clinical sign presented in groups A, B and C. Body weight was used as a primary measure of disease severity because of its importance in production and because it can be quantified. Differences were observed between the control group and the virus/bacteria exposed groups. Among the groups that were exposed, the group exposed to Rotavirus/Salmonella combination showed more clinical signs and lesions than others. Although differences among groups exposed to either virus or bacteria, or virus/bacteria combination were not significantly different from each other until about 42 dpi, the group that was exposed to virus/bacteria combination consistently had the lowest body weights. This is similar to the findings of Jindal *et al.* (2009) and Otto *et al.* (2006), in broilers with RSS. This observation was also reported by Guy *et al.* (2000), who observed high mortality and increased growth depression in turkey poults inoculated with turkey coronavirus and entero-pathogenic *E. coli* than those inoculated with turkey coronavirus or *E. coli* alone.

Intestinal lesions were frequent in the birds examined from inoculated groups but not in the controls. The observed lesions such as villous atrophy account for the clinical sign of diarrhoea described in rotavirus infection. Enteric lesions are commonly induced by enteropathic viruses (e.g., rotavirus, coronavirus, torovirus) in different animal species and humans (Mettifogo *et al*., 2014). These infectious agents have a tropism for the mature entero-absorptive cells lining the intestinal villi and destroy those cells during their replication. The lack of mature enterocytes decreases the absorption of nutrients and electrolytes as well as the digestion of nutrients by enzymes localized in the microvillous region of mature enterocytes thus causing malabsorption, maldigestion, and osmotic retention of water (Barker *et al.*, 1992). The severity of clinical signs may vary depending upon the extent of enterocyte loss. Perhaps, this is why group C birds

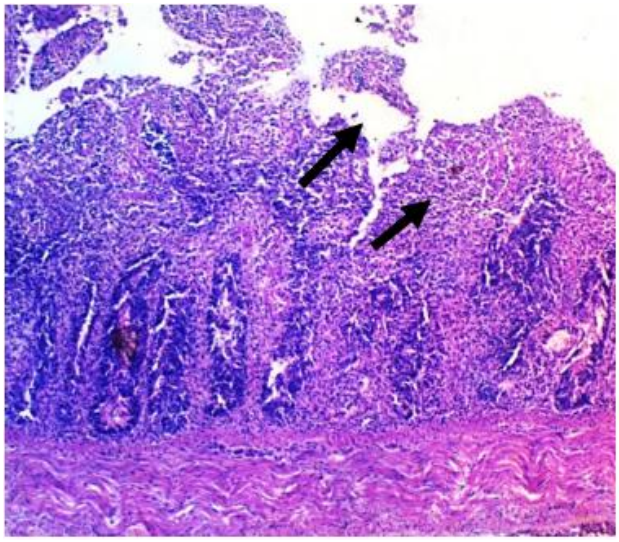

Plate III: Photomicrograph of intestine of group C bird infected with rotavirus and *Salmonella pullorum* showing severe necrosis of the crypts of lieberkuhn and the villi (arrow) with H&E stain at 14dpi. x150

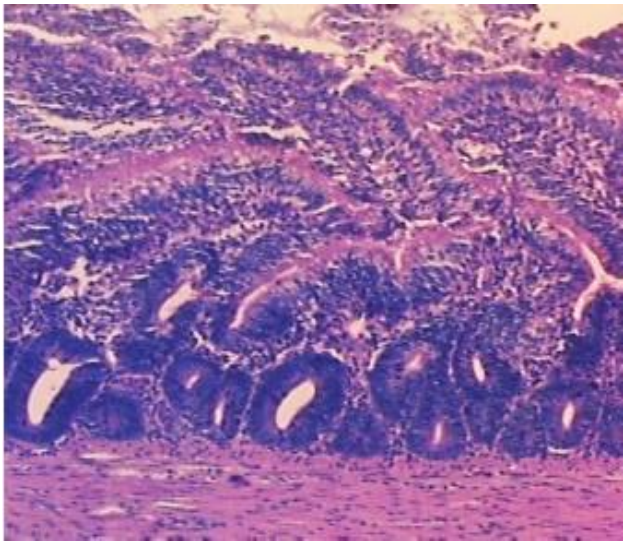

Plate IV: Photomicrograph of intestine of group D bird administered PBS showing normal crypts of lieberkuhn and enterocytes (H&E stain) at 14dpi. X350

had more diarrheic faeces than other groups because of the extent of their intestinal lesions (Plate III). In some cases, the lesions are not found throughout the small intestine but instead, a regional distribution in the middle-to-caudal small intestine and extensive sampling of the intestine will be necessary to identify them (Otto *et al.*, 2006). The clinical finding of diarrhoea as observed in the present study has been stated as a clinical sign of rotavirus infection (Dhama *et al*., 2015). These signs

were more prominent in the rotavirus/*Salmonella pullorum* infected group. It has also been reported in PD infection that birds manifest somnolescence, weakness, depressed appetite, poor growth, and adherence of chalky white material to the vent. In some cases, evidence of PD is not observed until 5-10 days and mortality may vary from 0 to 100%. Survivors may be greatly retarded in their growth and appear underdeveloped and poorly feathered. These birds may not mature into vigorous or well-developed laying or breeding birds (Jindal *et al.*, 2009).

The present study thus reveals that oral inoculation of birds with rotavirus or Rotavirus/*Salmonella pullorum* combination leads to diarrhoea and significant growth depression for up to 6 wk of age. Although infected ones that recover from diarrhoea may return to normal body weight after infection, the period to reach market weight would have been extended. The delayed weight gain is attributed to either a delayed or incomplete recovery of the intestinal mucosa or to the failure to compensate the transiently reduced weight gain during the short time span until slaughter. It is also important to note that severe clinical signs in rotavirus infection can also be due to the presence of another underlying infection from bacterial organism.

In Nigeria, broiler birds are raised up to 6 weeks of age for fast food restaurants or more for sale in the open market. For birds to meet market weights, they would have to be free of infections that would hinder their growth even when mortality is not recorded on the farm. Birds are bought based on their weight and not age, and owners of such birds would have spent a lot of money to reach market weight because of their high feed conversion ratio. It is thus important to continuously screen and prevent against organisms infecting the GIT for optimal performance of birds.

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