PRODUCTION AND TESTING THE EFFICACY OF LIVE ATTENUATED AND INACTIVATED VACCINES AGAINST EXPERIMENTAL *SALMONELLA* KENTUCKY INFECTION IN BROILER CHICKENS

BY

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DECLARATION

I hereby declare that the work in this dissertation entitled **"Production and Testing the Efficacy of Live Attenuated and Inactivated Vaccines against Experimental *Salmonella* Kentucky Infection in Broiler Chickens"** was carried out by me in the Department of Veterinary Microbiology, Ahmadu Bello University, Zaria, under the supervisions of Dr. K. A. Majiyagbe and Dr P. H. Mamman.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

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CERTIFICATION

This Dissertation entitled **"PRODUCTION AND TESTING THE EFFICACY OF LIVE ATTENUATED AND INACTIVATED VACCINES AGAINST EXPERIMENTAL *SALMONELLA* KENTUCKY INFECTION IN BROILER CHICKENS"** meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To my dear wife

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ABSTRACT

Salmonellosis is of major veterinary and public health importance. *Salmonella* Kentucky exhibits the potential to emerge as the most prominent *Salmonella* serotype in human disease. The aim of this study was to evaluate the efficacy of prepared live attenuated and inactivated vaccines against salmonellosis caused by *Salmonella* Kentucky. Field isolate of *Salmonella* Kentucky was obtained from the National Veterinary Research Institute Vom, Nigeria. Broiler chickens were purchased and ascertained to be free of Salmonellae and distributed into three experimental groups. 5-bromouracil (Sigma Aldrich) at a concentration of 1,000 μg/mL was used to attenuate the bacteria for the preparation of the live vaccine and 0.6 % formaldehyde (Sigma Aldrich) was used to inactivate the bacteria for preparation of the inactivated vaccine. The birds in the first group were immunized using the live attenuated vaccine administered subcutaneously at 0.5 mL per bird at day 11 of age and a booster of same dose was given at day 25. The birds in the second group received 1 mL of the inactivated vaccine subcutaneously at day 11 of age and a booster of same at day 25. Birds in the third group served as unvaccinated/control. The birds in both vaccinated and unvaccinated groups were challenged orally with 1 mL of the field isolate containing 5 x 10^7 CFU/mL of *Salmonella* Kentucky suspension at day 32 of age. There were no mortalities recorded in the immunized groups. The degree of protection was assessed in birds using the antibody (IgY) titre developed in serum and the caecal *Salmonella* Kentucky load present in faeces. Broiler chickens immunized with the live attenuated vaccine preparation at day 54 of age had no Salmonellae present in faeces, equivalent to 100 % inhibition and vaccinal efficacy. Also, broiler chickens immunized using the inactivated vaccine showed a decline in faecal Salmonellae shed; 82.82 % vaccinal efficacy at day 56 of age when compared with the control. Over all the faecal *Salmonella* count in Cloacal swab of birds immunized with

experimental vaccines at day 56 of age was lower than the unvaccinated group; 0 CFU/mL for the live attenuated vaccine and 3.5 x 10^2 for the inactivated vaccine groups while the control had 1.33 x 10^5 CFU/mL. The vaccinated birds also showed a higher antibody (IgY) titre levels from day 21 of age to day 56 of age ($P < 0.001$) on all sampling days when values were compared with the unvaccinated birds. In conclusion, the interplay between vaccination protocol that includes administration of live attenuated or inactivated *Salmonella* Kentucky vaccines and developed antibody (IgY) titre can reduce Caecal colonization by *Salmonella* Kentucky and shedding of the serovar. Further studies should be carried out on experimental vaccine to establish its safety and shelf life, also a detailed antibody response, induced cell mediated immunity and other complement cascade interactions be studied.

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LIST OF ABBREVIATIONS

5-BU	5-Bromouracil
APEC	Avian Pathogenic *Escherichia coli*
BA	Blood Agar
BGA	Brilliant Green Agar
CFU/mL	Colony Forming Unit per mL
CIPR	Ciprofloxacin Resistant
DMT	Drug Metabolite Transporter
ELISA	Enzyme Linked Immunosorbent Assay
EPS	Extracellular Polymeric Matrix
FL	Faecal Leukocytes
GALT	Gut Associated Lymphoid Tissue
HIV	Human Immunodeficiency Virus
IgY	Immunoglobulin Y
IL	Interleukin
iNTS	Invasive Non Typhoidal *Salmonella*
LAH	Lactalbumin Hydrolysate
LPS	Lipopolysaccharide
MATE	Multidrug and Toxic Compound Extrusion
MFS	Major Facilitator Superfamily.
MIC	Minimum Inhibitory Concentration
NB	Nutrient Broth
NS	Normal Saline
NTS	Non Typhoidal *Salmonella*
PAMP	Pathogen Associated Molecular Patterns
RBC	Red Blood Cell
PFGE	Pulsed Field Gel Electrophoresis

PRR	Pathogen Recognition Receptors
RND	Resistance- Nodulation-Division Superfamily.
RV	Rappaport Vassiliadis
SDA	Sabouraud Dextrose Agar
SMR	Small Multidrug Resistance
SPA	Serum Plate Agglutination
SPI	*Salmonella* Pathogenicity Island
T3SS	Type III Secretion System
TLR	Toll-Like Receptors
TSI	Triple Sugar Iron
WBC	White Blood Cell
XLD	Xylose Lysine Deoxycholate

CHAPTER ONE

INTRODUCTION

1.1 Background

Salmonellosis caused by *Salmonella* species is of major Veterinary and Public Health importance. It is also one of the most prevalent diseases in birds, causing high losses in the poultry industry with significant socio-economic impact (Metawe and Tulip, 2004; Foley *et al*., 2008). The genus consists of more than 2,600 different serovars and more than 50 serogroups described in the Kauffman White scheme and majority of the recognized serovars of *Salmonella* cause infections in both human and animals worldwide (Guibourdenche *et al*., 2010; Scallan *et al*., 2011; Issenhuth-Jeanjean *et al*., 2014). 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 (Majowicz *et al*., 2010; Fuche *et al*., 2016). Non typhoidal *Salmonella* can also cause severe extra-intestinal, invasive bacteremia, which is referred to as invasive non typhoidal salmonellosis (iNTS) (Feasey *et al*., 2012). Although more than 50 serogroups and 2,600 serovars have been described, not all *Salmonella* serovars cause disease in humans and animals. Efforts are being made to develop non typhoidal *Salmonella* vaccines, with most approaches eliciting protection against serovars Typhimurium and Enteritidis (serogroups B [O: 4] and D [O: 9], respectively), as they are widely considered the most prevalent (Fuche *et al*., 2016). Serogroup C (O:6,7, O:6,8, or O:8 epitopes) is now the most common serogroup in the United States, and the prevalence of serovars from this serogroup has been increasing in Europe and the United States over the last decade. They are also the most commonly isolated serovars from healthy cattle and poultry, indicating the underlying importance of surveillance in animals. Invasive non-typhoidal *Salmonella* commonly causes

infection in children, the elderly, infants and immuno-compromised individuals worldwide and especially in Africa where the disease is driven in part by co-infection with malaria or HIV (Ao *et al*., 2015). Poultry meat is a major source of animal protein in Nigeria as well as other developing countries because it is readily available, affordable and acceptable (Bettridge *et al*., 2014). Poultry farming is threatened by salmonellosis and avian influenza (FAO, 2006). A reduction in the burden of salmonellosis in humans involves the elimination of the infection from livestock (Snow *et al*., 2010), this may be achieved through understanding the factors associated with the spread of the infection (Snow *et al*., 2010).

1.2 Statement of the Research Problem

Over the last several decades, there have been significant shifts in the predominant *Salmonella* serovar associated with poultry and human infections (CDC, 2008). *Salmonella enterica* serovars Gallinarum and Pullorum caused widespread diseases in poultry in the early 1990s. Vaccination and biosecurity programmes have helped eradicate fowl typhoid and pullorum from commercial flocks in some developed countries (Foley *et al*., 2011). One potential consequence of eradicating *S.* Gallinarum in poultry was the emergence of *S.* Enteritidis (Foley *et al*., 2011). Prior to the emerging increase in *S.* Enteritidis infection in chickens, this serovar was associated primarily with rodents (Callaway *et al*., 2008). While *S.* Enteritidis has remained a significant problem in commercial poultry, the prevalence of this serovar has declined in chickens since the mid 1990s. *S.* Heidelberg supplanted *S.* Enteritidis as the predominant serovar from 1997 to 2006, but in 2007, *S.* Kentucky was the most frequently isolated serovar in the United States (Foley *et al*., 2008). This changing trend could be because as immune response are continually

and specifically directed at *S.* Enteritidis surface antigen, the prevalence of *S.* Heidelberg and *S.* Kentucky potentially increased in the absence of *S.* Enteritidis (Brenner, 1998). In the past, *S.* Kentucky was less often identified in human salmonellosis, averaging 62 cases per annum from 1996 to 2004 in the United States, but rose to 123 cases in 2006 (CDC, 2008), accordingly, the prevalence of *S.* Kentucky in chickens had increased from 25 % in 1997 to approximately 50 % in 2007 in the United States (Fricke *et al*., 2009; FDA, 2010). Four out of the ten most lethal serovars in the United States are serogroup C. Serogroup C consists of highly diverse organisms among which 37 serovars account for the majority of human cases, compared to 17 and 11 serovars for serogroups B and D, respectively (Parry *et al*., 2013). Johnson *et al.* in 2010 reported that *S.* Kentucky harbors a *ColV* virulence plasmid gene which significantly increased its ability to colonize the chicken caecum and cause extra-intestinal disease; in the liver, pericardium and air sac, following experimental subcutaneous injection and bacteria recovery from these organs in birds. *Salmonella* Kentucky isolates were noted, and specifically a ciprofloxacin-resistant (and in some cases carbapenemase containing) clone to cause concerns (Le Hello *et al*., 2013b). Although first being considered as a travel-related clone, it seems to have spread to poultry and the environment (Le Hello *et al*., 2013b). This population was mostly detected in Egypt before 2005, but has now rapidly spread throughout Africa and the Middle East (Le Hello *et al*., 2011). This emerging *S.* Kentucky strain has accumulated various chromosomal resistance determinants since the mid 2000s, encoding resistance to multiple antimicrobials including amoxicillin, gentamicin, and sulphonamides (Boyd *et al.*, 2001; Weill *et al*., 2006; Doublet *et al*., 2008; Le Hello *et al*., 2011; Le Hello *et al*., 2013a), followed by cumulative mutations in the *gyrA* and *parC* genes, leading to resistance to nalidixic acid and then to ciprofloxacin, causing infection in humans probably through contaminated imported foods or

secondary contaminations (Le Hello *et al*., 2011). Another matter of concern is the expanding livestock reservoir of the strain (*S.* Kentucky *CIP-R*), initially identified in African autochthonous poultry (Chickens), has subsequently been found in various animals and food (Le Hello *et al*., 2011; Beutlich *et al*., 2012; Münch *et al*., 2012; Wasyl and Hoszowski, 2012; Barua *et al*., 2013). These constant threats that arise from sudden increase in emergence and prevalence of new serotypes are often due to unknown or unclear reasons (Van Immerseel, 2016). Proposed factor that may contribute to the potential emergence of *S.* Kentucky is the acquisition of virulence plasmids from avian pathogenic *Escherichia coli* (APEC*)* (Fricke *et al*., 2009; Johnson *et al*., 2010). A large percentage of *S.* Kentucky strains isolated from chickens carry these plasmids; this also seems to be an important factor to both APEC and *S.* Kentucky for colonization of poultry (Bohez *et al*., 2006). Poultry flocks have contributed to the wide spread of this clone in developing countries since 2005 as this strain was isolated in Ethiopia in 2006 (chicken), Nigeria (chicken) and Morocco (turkey) in 2008, Togo (chicken) in 2009 (Le Hello *et al*., 2013a). In Tunisia between 2005 and 2008, a total of 57 *S.* Kentucky strains were isolated from different sources and were characterized by their antimicrobial and mercury resistance profiles; ability to form a biofilm; virulence *invA*/*spvC* genes and quorum sensing *sdiA* gene (Turki *et al*., 2012). A total of 10.6 % of the isolates demonstrated multidrug-resistance against 3 to 13 antibiotics with ciprofloxacin resistance occurring in 33 % of human isolates. In addition, 37 % of the isolates exhibited minimum inhibitory concentrations value to mercuric chloride, ranging from 8 to 32 $\mu g ml^{-1}$ and were considered as resistant strains (Turki *et al*., 2012). In Egypt *Salmonella* Kentucky isolated showed high degree of antibiotic resistance especially toward ciprofloxacin which is considered the drug of choice for *Salmonella* treatment (Nourhan *et al*., 2014). In Nigeria there is a high rate of consumption of poultry products and an increase in

the cases of *Salmonella*-associated gastroenteritis is frequent (Fasure *et al*., 2012). The use of antimicrobials in any environment creates selection pressures that favours the survival of antibiotic-resistant strains (Adeleke *et al*., 2012) and in Nigeria, the practice of routinely giving antimicrobials to domestic livestock for growth promotion and prophylaxis is an important factor in the emergence of antibiotic-resistant bacteria in the food chain (Fasure *et al*., 2012; Shah and Korejo, 2012).

1.3 Justification for the Study

In a recent Survey in Nigeria, *S.* Kentucky was the most prevalent serotype (16.2 %) followed by *S*. Poona (5.66%) and *S.* Elisabethville. Also, *S.* Kentucky was distributed across the country as samples were drawn from farms across six states in the six geopolitical zones in Nigeria (Fagbamila *et al*., 2017). Mshelbwala *et al*. (2017) reported *Salmonella* Kentucky as one of the *Salmonella* serotypes isolated in poultry farms in three south western states in Nigeria with 24.32 % prevalence. In another study carried out in South western and North central Nigeria, Useh *et al*. (2016), established that *S.* Kentucky, was the common serotype isolated from poultry followed by *S.* Zega and *S.* Hertston, and though *S. enterica* serotype Enteritidis is the most common serotype associated with poultry worldwide, it was absent in his study. Also, an investigative study on the antimicrobial resistance and clonality of *Salmonella enterica* serotype Kentucky isolates from chicken and farm environmental sources (litter, faeces, water) in Ibadan and Maiduguri Nigeria was conducted. Isolates were screened to establish for the presence of ciprofloxacin resistant genes (*S.* Kentucky *CIPR* strain) using PFGE and MIC in 2007, 2010 and 2011. Six per cent of the samples were positive for *S.* Kentucky and all resistant to nalidixic acid and ciprofloxacin (Raufu *et al*., 2014). The isolates showed genotypic relatedness to *S.* Kentucky

CIPR strain based on *Xba*I PFGE fingerprinting, indicating the presence of the widely spread *CIPR S.* Kentucky clone within poultry and farm environment in this regions (Raufu *et al*., 2014). These factors express the likelihood of a widespread virulent *CIPR* antibiotic resistant strain of *S.* Kentucky in Nigeria. Despite these concerning data, no human vaccines targeting serogroup C NTS are available and animal vaccines are in limited use (Fuche *et al*., 2016). While the virulence and colonization mechanism of *S.* Enteritidis and *S.* Typhimurium have been extensively studied, little work on *S.* Kentucky and *S.* Heidelberg has been done (Foley *et al*., 2011). Vaccination has shown an unrivalled impact on global health (Plotkin, 2009) and vaccines have the greatest potential for further improvement in health in the poorest countries of the world (MacLennan, 2013) and these comprise both live and inactivated vaccines. Currently available live vaccines against *Salmonella* are produced by chemical mutagenesis or are selected on culture media as slow growing natural mutants (metabolic drift mutants) (Van Immerseel, 2016). 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 (Gast, 2007). A lot of experimental vaccines against Salmonellosis have been produced for chickens, and a variety of commercial vaccines are also available in the market (Van Immerseel, 2016), but vaccines against *S.* Kentucky is rare. In order to reduce the spread of the circulating virulent strains of *S.* Kentucky in birds and its subsequent transmission to man, this study proposes to produce a trial live attenuated and inactivated *S.* Kentucky vaccines for birds, thereby improving humoral immunity in birds to prevent and reduce caecal and intestinal colonization by the virulent strain.

1.4 Aim of the Study

To evaluate the efficacy of prepared live attenuated and inactivated *Salmonella* Kentucky experimental vaccines in preventing caecal colonization of the serovar in broiler chickens.

1.5 Objectives of the Study

1. To produce a live attenuated *Salmonella* Kentucky vaccine using 5-bromouracil.
2. To produce an inactivated *Salmonella* Kentucky vaccine using 0.6 % formalin.
3. To determine the antibody titre level in serum of broilers following vaccination with the live attenuated and inactivated *S.* Kentucky vaccines.
4. To determine the level of *S.* Kentucky serovar shedding in faeces of broilers following administration of live attenuated and inactivated *S.* Kentucky vaccines.

1.6 Research Questions

1. Can 5-bromouracil attenuate *Salmonella* Kentucky?
2. Can 0.6 % formalin inactivate *Salmonella* Kentucky?
3. Could vaccination of broiler chickens using trial attenuated and inactivated *S.* Kentucky vaccines elicit adequate humoral response?
4. Could vaccination of broiler chickens using trial attenuated and inactivated *S.* Kentucky vaccines reduce faecal shedding in broiler chickens?

CHAPTER TWO

LITERATURE REVIEW

2.1 Antimicrobial resistance in *Salmonella enterica* infections

Salmonella enterica infections are common causes of bloodstream infections in low resource areas, where they may be difficult to distinguish from other febrile illnesses and may be associated with high case fatality. Microbiologic culture of blood or bone marrow remains the mainstay of laboratory diagnosis. Antimicrobial resistance has emerged in *Salmonella enterica,* initially to the traditional first line drugs, chloramphenicol, ampilcillin and trimethoprim-sulfamethoxazole. Decreased flouroquinolones susceptibility and then flouroquinolones resistance have developed in association with chromosomal mutations in the quinolone resistance determining region of genes encoding DNA gyrase and topoisomerase IV and also by plasmid mediated resistance mechanisms. Resistance to extended spectrum cephalosporins has occurred more often in nontyphoidal than typhoidal *Salmonella* strains. Azithromycin is effective for the management of uncomplicated typhoid fever and may serve as an alternative oral drug in areas where fluoroquinolone resistance is common (Crump *et al*., 2015). In 2013, CLSI (Clinical and Laboratory Standard Institute) lowered the ciprofloxacin susceptibility breakpoints to account for accumulating clinical, microbiologic and pharmacokinetic-pharmacodynamic data suggesting that revision was needed for contemporary invasive *Salmonella* infections (Crump *et al*., 2015). *Salmonella enterica* serotype Kentucky (*S.* Kentucky) has been closely associated with poultry since it was first isolated in 1937 from a chick in the United States of America (USA) (Crump *et al*., 2015).

2.2 History and Taxonomy of *Salmonella* spp

Salmonella was named after Daniel Salmon an American, a veterinary pathologist while Theobald Smith also American was the discoverer of the type bacterium (*Salmonella enterica* var Cholerasuis) in pigs (FDA, 2008). In 1900, Lignieresi an Administrator of the United States Department of Agriculture (USDA) Research Program in honour of Dr. Daniel Salmon gave the generic name to this bacterium *"Salmonella"* (FDA, 2008). *Salmonella* belongs to the kingdom: Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Enterobacteriales, Family: Enterobacteriaceae and Genus: *Salmonella* (Fox, 2011). Other groups of the family Enterobacteriaceae to which *Salmonella* belongs are: *Citrobacter, Escherichia, Edwardsiella, Enterobacter, Ewingella, Hafnia, Klebsiella, Kluyvera, Morganella, Proteus, Providencia, Serratia, Shigella, Tatumella and Yesinia* (Fox, 2011). The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*. The species *Salmonella enterica* is further subdivided into six subspecies that are designated by taxonomic names; these are sometimes abbreviated by Roman numerals; subspecies I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), VI (*indica*) (CDC, 2011).

2.3 Microbiology of *Salmonella* spp

2.3.1 Culture media for Isolation of *Salmonella* spp

Salmonella requires selective enrichment broths which are formulated to selectively inhibit other bacteria while allowing it to multiply to levels that may be detected after plating. There are currently three major types of selective enrichment broth media namely; tetrathionate, Selenite and Rappaport-Vassiliadis (RV). There are also different formulations within each type of

enrichment (The Oxoid, 2006). Selective plating media such as *Salmonella-Shigella* agar, MacConkey, Bismuth sulphite, Brilliant green agar (BGA) and brilliant green sulphapyridine agar, Xylose lysine Deoxycholate (XLD) and Deoxycholate Citrate lactose sucrose agar are used (OIE, 2008). This is due to high levels of non *Salmonella* bacteria found in intestinal and environment samples. Such media are used to assist the selective enrichment broth to inhibit other bacteria (Waltman *et al*., 1998). *Salmonella* spp. are aerobic to facultative anaerobes. The optimum temperature for growth is 37 ^{o}C, but some can grow at a temperature range of 5 ^{o}C – 45 ^{o}C. The pH for growth is 4 to 9 (Gast and Beard, 1990).

2.3.2 Colonial Morphology

Typical *Salmonella* colonies on agar media are about 2 – 4 mm in diametre with round smooth edges, slightly raised and glistering. On blood agar, they form moist colonies at 2 – 3 mm in diameter. They appear red to pink on brilliant green agar, Brilliant sulphapyridine and brilliant green novobiocin, black metallic sheen on bismuthsulphite agar, red/pink with black center on Xylose Lysine Tergitol 4 and Xylose Lysine Deoxycholate agar, colourless with black center on deoxycholate agar and *Salmonella-shigella* agar, Blue-green with black center on Hecktoen enteric agar, colourless on MacConkey, crimson with pale borders on Rambach agar (Waltman *et al*., 1998). The viability of *Salmonella* can be maintained for a year in simple media such as peptone and nutrient agar (Gast and Beard, 1990).

2.3.3 Biochemical properties of *Salmonella* spp

Typical *Salmonella* colonies found on the selective agar should be confirmed by biochemical tests. The principal biochemical tests by which *Salmonella* can be identified are as follows: Nitrate reduction, glucose, oxidase, mannitol, oxidation/fermentation, maltose, urea hydrolysis, lactose, sucrose, salicin, dulcitol, adonitol, indole, H_2S production, citrate utilization, sodium malonate, growth on lysine decarboxylase, methyl red, Voges Proskauer, arginine dihydrolase, ornithine decarboxylase, gelatin liquefaction. Testing all these reaction requires considerable resources. Therefore, it makes sense to use composite media such as triple sugar iron (TSI) agar which is prepared in tubes for further use. It contains glucose, lactose and sucrose, a H_2S detection system and an indicator (Jones *et al*., 2000). When glucose not lactose or sucrose is fermented by the microorganisms a change of colour from red to yellow can be observed. The colour of fermentation reaction depends on the amount of glucose which is converted. However, the reaction under aerobic conditions on the slant reverts and become alkaline (red) because of protein break down in the medium. Under anaerobic condition inside the agar at the bottom of the tube, the medium remains acidic (yellow) and H_2S production is characterized by blackening of the medium (Jones *et al*., 2000). Some *Proteus* Spp. and other species may give a similar reaction, but can be distinguish by their ability to hydrolyze urea. Urea agar in another reaction tube should always be used in parallel with TSI. The common bacterial characters of *Salmonella* are as follows; Fermentation of glucose, maltose, mannitol and sorbitol with the production of acid and gas. Absence of fermentation of lactose, sucrose, salicin and adonitol. Failure to produce indole, to hydrolyze urea, to deaminate phenyl alanine, to use malonate, or grow in potassium cyanide medium (Jones *et al*., 2000).

2.4 *Salmonella* Infections

2.4.1 Human Infections

Salmonella enterica infections are a significant public health concern in many low and middle income countries (Reddy *et al*., 2010; Deen *et al*., 2012). *Salmonella* Typhi and *Salmonella* Paratyphi A (*S.* Typhi, *S.* Paratyphi), collectively known as typhoidal *Salmonella*, are causal agents for a serious, invasive (bacteraemic), sometimes fatal disease of humans called typhoid fever or paratyphoid fever (also called enteric fevers) (Sanderson *et al*., 2015). *Salmonella* Typhi, the lineage causing typhoid fever, is the main group; *Salmonella* Paratyphi A, the lineage causing paratyphoid fever, belongs to the second group which comprises a set of three paratyphoid types (the other two being *S.* Paratyphi C and d-tartrate negative *S.* Paratyphi B). Non typhoidal *Salmonella* (NTS) strains are not host specific, they infect or colonize a broad range of vertebrate animals and all of these lineages are adapted to humans (Sanderson *et al*., 2015) or may be adapted or restricted to particular non human animal species (Feasy *et al*., 2012) Symptoms of infection are a gradual rise in temperature, typically worse through the day, over the first 2 – 3 days reaching 40 ^{o}C and often swinging, relative bradycardia (Faget's sign); the pulse is slower than would be expected from the degree of temperature. Malaise and weakness, headache, abdominal pain, diarrhoea and constipation may also occur, hepatosplenomegaly is common. In this bacteraemic phase it is possible to find bacteria in the reticuloendothelial tissues (Nordqvist, 2016) and as the disease progresses cholecystitis, endocarditis, Osteitis, encepahalitis especially in infants, dehydration (which is a significant risk) one third develop a macular truncal rash, thrombocytopenia with risk of internal bleeding, rupturing or splitting of the bowel, myocarditis (inflammation of the myocardium, the heart muscle), multiple organ failure as the bacteria start releasing toxins (Nordqvist, 2016). *Salmonella* produces both endotoxins and

exotoxins. The exotoxins can be subdivided in two types; the cytotoxins and the enterotoxins. Cytotoxins also called verotoxins; referring to a cytotoxic assay in Vero cells are defined by their ability to kill mammalian cells (Alphons and Jaap-van Dijk, 2005). All tested strains of serotypes *S.* Cholerasius (25 strains), *S.* Enteritidis (94 strains) as well as *S.* Typhi (12 strains) produce a heat labile, trypsin-sensitive cytotoxin (Alphons and Jaap-van Dijk, 2005). After ingestion, infection with *Salmonellae* is characterized by attachment of the bacteria by fimbriae or pilli to cells lining the intestinal lumen. *Salmonella* selectively attach to specialized epithelial cells (M cells) of the Peyer's patches. The bacteria are then internalized by receptor mediated endocytosis and transported within phagosomes to the lamina propria, where they are released. Once there, *Salmonella* induce an influx of macrophages (Typhoidal strains) or neutrophils (non typhoidal strains) (Klochko, 2016). Typhoid and Paratyphoid fever usually present as clinically similar acute febrile illnesses; accurate diagnosis relies on confirmation by laboratory tests (Sanderson *et al*., 2015). The Vi antigen of *S.* Typhi is important in preventing antibody mediated opsonization and complemant mediated lysis. Through the induction of cytokine release and via mononuclear cell migration, *S.* Typhi organisms spread through the reticuloendothelial system, mainly to the liver, spleen and bone marrow. Within 14 days, the bacteria appear in the bloodstream, facilitating secondary metastatic foci (eg, splenic abscess, endocarditis). In some patients, gallbladder infection leads to long term carriage of *S.* Typhi or *S.* Paratyphi in bile and secretion to the stool (Meltzer and Schwartz, 2007). As a rule, infection with non typhoidal *Salmonella* generally precipitates a localized response, while *S.* Typhi and other especially virulent strains invade deeper tissues via lymphatics and capillaries and elicit a major immune response (Klochko, 2016). Some risk factors for Salmonellosis were found to be corticosteroid use, malignancy, diabetes, HIV infection, prior antimicrobial therapy, and immunosuppressive

therapy (Klochko, 2016). Infants are at a high risk of developing CNS (Central nervous system) infection as a result of *Salmonella* bacteremia. Specific anatomical sites, such as an altered urinary or biliary tract, atherosclerotic aorta, or endovascular devices may facilitate persistent focal *Salmonella* infection (Klochko, 2016).

2.4.1.1 Nontyphoidal Salmonella

Nontyphoidal *Salmonella* (NTS; i.e., *Salmonella enterica* organisms that do not cause typhoid or paratyphoid) are responsible for 94 million infections and 155,000 deaths worldwide annually, 86% of which are estimated to be foodborne (Majowicz *et al*., 2010). Although more than 50 serogroups and 2,600 serovars have been described (Guibourdenche *et al*., 2010; Scallan *et al*., 2011; Issenhuth-Jeanjean *et al*., 2014), not all *Salmonella* serovars cause disease in humans and animals. Efforts are being made to develop NTS vaccines, with most approaches eliciting protection against serovars Typhimurium and Enteritidis (serogroups B [O:4] and D [O:9], respectively), as they are widely considered the most prevalent (Fuche *et al*., 2016). Serogroup C (O:6,7, O:6,8, or O:8 epitopes) is currently the most common serogroup in the United States, and the prevalence of serovars from this serogroup has been increasing in Europe and the United States over the last decade. They are also the most commonly isolated serovars from healthy cattle and poultry, indicating the underlying importance of surveillance in animals. Four out of the ten most lethal serovars in the United States are serogroup C, and reports from African countries suggest that strains within this serogroup are highly antibiotic resistant (Fuche *et al*., 2016). Serogroup C consists of highly diverse organisms among which 37 serovars account for the majority of human cases, compared to 17 and 11 serovars for serogroups B and D,

respectively. Despite these concerning data, no human vaccines targeting serogroup C nontyphoidal salmonellosis are available and animal vaccines are in limited use. In industrialized countries, nontyphoidal *Salmonella* is transmitted predominantly by commercially produced food contaminated by animal faeces, and it usually causes a self-limited enterocolitis with diarrhoea in humans. Bloodstream infection occurs in approximately 6% of patients with diarrhoea enterocolitis; infants, young children, the elderly, and the immunocompromised are at particular risk for bacteremia (Parry *et al*., 2013). Nontyphoidal *Salmonella* serovars are diverse in their host range and epidemiology and vary in their propensity to cause bloodstream infection and severe human disease. *Salmonella enterica* serovar Typhimurium is considered to be a typical example with a broad host range and likelihood of causing invasive disease, while other nontyphoidal *Salmonella* serovars such as *Salmonella* serovars Heidelberg, Dublin, and Choleraesuis are markedly more likely than *Salmonella* serovars Typhimurium to cause hospitalization invasive disease, or death. Some serovars, such as *Salmonella* serovars Newport, are associated with a lower case fatality rate (0.3%) than *Salmonella* serovars Typhimurium (Jones *et al*., 2008; Parry *et al*., 2013). The reasons for differences in host specificity among *Salmonella* serovars are complex and incompletely understood (Barrow, 2007). Typhoidal *Salmonella* serovars results in an immuno-suppressive response compared with nontyphoidal *Salmonella* serovars associated with diarrhea and also produce a unique exotoxin, which may explain differences in the clinical phenotype (Jones *et al*., 2008; Gal-mor *et al*., 2014). Modes of nontyphoidal *Salmonella* transmission in low and middle income countries are less well understood than in industrialized countries. Malaria and malnutrition predispose to invasive disease among children, and HIV does so among both children and the young (Feasy *et al*., 2012). Worldwide in 2006, enteric nontyphoidal *Salmonella* was estimated to cause 93.8 million

diarrheal illnesses and 155,000 deaths (Majowicz *et al*., 2010). In the institute for Health Metrics and Evaluation Global Burden of Disease 2010 project, enteric nontyphoidal *Salmonella* was estimated to account for 4.8 million disability-adjusted life years (Murray *et al*., 2012) and 81,300 deaths (Crump *et al*., 2015). In 2010, nontyphoidal *Salmonella* was estimated to cause approximately 3.4 million invasive infection and 681,000 deaths; 57% of these illnesses and deaths occurrence in Africa (Ao *et al*., 2015). The most widely reported serovars associated with invasive disease across Africa are *Salmonella* serovar Typhimurium and *Salmonella* serovar Enteritidis (Gordon *et al*., 2010; Crump *et al*., 2015). A novel *Salmonella* serovar Typhimurium multilocus sequence type, ST313, has been described and currently accounts for much invasive disease in Sub-Saharan Africa. This sequence has a unique prophage repertoire and a degraded genome that shows some convergence with that of *Salmonella* serovar Typhi, raising the possibility of increased host specialization or invasiveness (Kingsley *et al*., 2009). One putative virulence gene, ST313td, has been described in *Salmonella* serovar Typhimurium ST313 and also found in other human invasive and enteric pathovars, notably *Salmonella* serovars Dublin (Herrero-fresno *et al*., 2014). However, to date no other candidate virulence genes have been associated with invasive nontyphoidal *Salmonella* strains from HIV-infected patients (Preziosi *et al*., 2012). Ongoing genomic and phenotype investigations of *Salmonella* serovars Typhimurium ST313 from Africa may be an area promising further new insights.

2.4.1.2 Sources and Mode of Transmission

While sources and modes of transmission of enteric nontyphoidal *Salmonella* have been studied extensively in industrialized countries (Painter *et al*., 2013), epidemiologic studies of

nontyphoidal *Salmonella* infections in area of endemicity in Sub-Saharan Africa are very limited (Morpeth *et al*., 2009). In industrialized countries, animal products and produce contaminated with animal faeces are important sources of nontyphoidal *Salmonella* (Painter *et al*., 2013). Contacts with animals, such as reptiles and with animal environments are important sources of nontyphoidal *Salmonella* infection (CDC, 2011). Although transmission by food contaminated with animal faeces is mostly considered, transmission directly from contact with animals and their environment, transmission between people, transmission independent of a nonhuman animal reservoir and waterborne transmission have been hypothesized. Partial genome molecular studies of nontyphoidal *Salmonella* strains from invasive human infections and those carried by animals among the households of children with invasive disease in Africa have so far failed to find similarities between strains, while family members of index cases have been found to have more closely related isolates (Kariuki *et al*., 2006). Although it was hypothesized that the degraded genome of *Salmonella* serovar Typhimurium ST313 might reflect a reduced host range and human restriction of the sequence type, recent studies have indicated that ST313 also displays a severe invasive phenotype in chickens but reduced potential for caecal colonization (Parsons *et al*., 2013). This suggests that transmission among humans may have exerted genomic selection pressure. Little is known about the epidemiology or phenotype of prevalent enteric nontyphoidal *Salmonella* strains in Africa. Nontyphoidal *Salmonella* was not a common cause of moderate to severe diarrhoeal disease in African sites participating in the Global Enteric Multicentre Study (Crump *et al*., 2015). However, asymptomatic carriage of nontyphoidal *Salmonella* appears to be relatively common (Kariuki *et al*., 2006).

2.4.1.3 Host risk and protective factors

Previous gastric surgery, pernicious anemia, and medications that reduce the acid barrier, such as antacids, H2 antagonists, and proton pump inhibitors, increase susceptibility to nontyphoidal *Salmonella* enteric infection (Bavishi and Dupont, 2011). Individuals at the extremes of age are at increased risk of invasive nontyphoidal *Salmonella*. In case of older people, this may be because of multiple co-morbidities, including diabetes, renal disease, or medications. The recent use of antimicrobials is associated with increased risk of multidrug-resistant *Salmonella* infection (Crump *et al*., 2015). In Africa, there is a clear bimodal age distribution that contrasts with the continuous age distribution through childhood to adulthood of typhoid fever. Children aged from 6 to 18 months and adults aged 25 to 40 years, in whom HIV prevalence is also highest, show the highest incidences of invasive nontyphoidal *Salmonella* in Africa (Feasy *et al*., 2010). Among children, there is relatively low incidence of invasive nontyphoidal *Salmonella* below the age of 6 months. This may be attributable to protection afforded by breastfeeding, by lack of exposure to contaminated water or food during exclusive breastfeeding, or by transplacental transfer protective IgG (MacLennan *et al*., 2008). However, neonatal invasive nontyphoidal *Salmonella* does occur, particularly among children born outside a health care facility (Milledge *et al*., 2005; Talbert *et al*., 2010).

Unlike enteric fever, for which where there are no clear clinical associations with classic immune-compromising conditions, invasive nontyphoidal *Salmonella* is associated with many forms of immune-compromise (Gordon, 2008). These include disorders of oxidative cellular killing, such as chronic granulomatous disease, in which nontyphoidal *Salmonella* is described as the most common cause of bloodstream infection and the third leading cause of infections (Crump *et al*., 2015). Children who are homozygous for sickle cell disease are susceptible to

invasive nontyphoidal *Salmonella* infection (Sommet *et al*., 2013). There is an overwhelming association of invasive nontyphoidal *Salmonella* with advanced HIV disease among African adults, with > 95 % of case being HIV infected (Reddy *et al*., 2010; Crump *et al*., 2015). Among children, HIV is a risk factor for infection with NTS. In cohorts of African children with invasive nontyphoidal *Salmonella*, around 20 % are typically HIV infected (Crump *et al*., 2015). Several immune defects have been described that could contribute to the apparent exquisite susceptibility of adults with advanced HIV to recurrent invasive nontyphoidal *Salmonella*. These include the loss of IL-17-producing CD4 cells in the gut mucosa, permitting rapid invasion (Crump *et al*., 2015), and dysregulated excess production of anti-LPS IgG that inhibits serum killing of extracellular *Salmonella* in a concentration-dependent fashion (MacLennan *et al*., 2010; Trebicka *et al*., 2014) Nontyphoidal *Salmonella* establishes an intracellular niche during invasive disease in HIV infection (Gordon *et al*., 2010). It is likely that this is facilitated by the ability of the bacteria to be rapidly internalized before serum killing can occur (Siggins *et al*., 2014). Once in the intracellular niche, reduction and dysregulation of proinflammatory cytokine responses in HIV-infection individuals, observed in vivo and ex vivo (Gordon *et al*., 2010), allow intracellular survival and persistence, leading to frequent recrudescence and relapses of bacteremia with identical strains of nontyphoidal *Salmonella* (Okoro *et al*., 2012). In contrast with HIV-infected adults, a lack of protective antibody appears to be implicated in the susceptibility of African children less than 18 months of age to invasive nontyphoidal *Salmonella* disease, and antibody is likely to be important both for cellular and cell-free control of nontyphoidal *Salmonella* disease in children (MacLennan *et al*., 2008). An association of invasive nontyphoidal *Salmonella* with malaria among African children was first noted in 1987 in West Africa. Since then, recent malaria, acute severe malaria (Crump *et al*., 2015), and severe malarial anemia, but not cerebral

malaria (Bronzan *et al*., 2007), have all been specifically described as risk factors for invasive nontyphoidal *Salmonella*. Malnutrition is also associated with invasive nontyphoidal *Salmonella* in African children (Crump *et al*., 2015). In rural Kenya, nontyphoidal *Salmonella* bacteremia was associated with child malnutrition, with an odds ratio (95 % confidence interval) of 1.68 (1.15 to 2.44) (Crump *et al*., 2015).

2.4.1.4 Presenting symptoms and signs

The clinical presentation of invasive nontyphoidal *Salmonella* infection is nonspecific among both children and adults. Therefore, recognition and management are challenging, particularly in settings without facilities for the laboratory diagnosis of bloodstream infection. Invasive nontyphoidal *Salmonella* presents as a febrile illness. Respiratory symptoms are frequently present, and diarrhoea is often not a prominent feature. However, often the clinician is faced with a febrile patient without an obvious clinical focus of infection. Features on physical examination include abnormal respiratory findings, such as rapid respiratory rate or chest crepitations suggestive of pneumonia, and hepatosplenomegaly in 30 % to 45 % of cases (Mandomando *et al*., 2009). Splenomegaly has been shown to be useful clinical feature to predict nontyphoidal *Salmonella* bacteremia among adults in areas where HIV seroprevalence is high (Crump *et al*., 2015). Patients with invasive nontyphoidal *Salmonella* also often display the features of underlying conditions such as anaemia, malnutrition, in advanced HIV disease. Nontyphoidal *Salmonella* meningitis may occur among children. It has poor outcomes and a high case fatality ratio (Molynuex *et al*., 2009). Although lower respiratory tract infection-related symptoms and signs are common in invasive nontyphoidal *Salmonella* infection, these are frequently caused by

other pathogens (Crump *et al*., 2015). Lobar pneumonia caused by nontyphoidal *Salmonella* has been described in a child (Mankhambo *et al*., 2006).

Mortality from invasive nontyphoidal *Salmonella* infection is high in all subgroups, even if appropriate antimicrobial therapy is given. Case fatality ratios among HIV-infected adults from African case series were ≥ 50 % early in the HIV epidemic (Crump *et al*., 2015). A study from Thailand reported a case fatality ratio of 36 % among all cases of invasive nontyphoidal *Salmonella* but 59 % among cases who were HIV infected. The reported case fatality ratio among adults appear to have fallen gradually in Africa, after initial reports in the 1980s and 1990s, to around 20 % to 25 % more recently. This has been attributed largely to improved recognition and more prompt and effective management (Crump *et al*., 2015).

2.4.2 Avian Infection

Salmonella is common in galliform birds, and has been isolated at high rates from commercially reared chicken, turkeys and other poultry. Apart from the associated foodborne risk, farms may represent a direct risk to public health, even though relevant studies are so far missing and high biosecurity standards in most commercial poultry farms minimizes the risk (Hoelzer *et al*., 2011). The clinical symptoms associated with *Salmonella* infection vary considerably by age and serotype (Hoelzer *et al*., 2011). Infections with generalist serotypes rarely cause clinical disease in galliform birds and most animals become asymptomatic carriers, even though severe clinical disease with high mortality has been observed in some instances, particularly during infections of young birds (Hoelzer *et al*., 2011). *Salmonella* Gallinarum with the biotypes Gallinarum and Pullorum causes two septicemic diseases in poultry. The later *Salmonella* Pullorum serovar is not

recognised anymore as such, both biotypes are differentiated by a few biochemical and molecular tests. This bacterium is highly adapted to produce a septicemic disease in birds. In general all other animal species are refractory to the disease (Terzolo and Chacana, 2016). Infections with the host adapted serotype Gallinarum biovars Gallinarum and Pullorum; however cause severe disease with high mortality with immense economic losses on chicken and turkey (Shivaprasad, 2000). *Salmonella* Gallinarum biovar Pullorum causes "Pullorum disease" in young animals, which is associated with septicemia and high mortality that can exceed 85% in some instances (Hoelzer *et al*., 2011). While in adult birds Pullorum disease is generally mild or asymptomatic, even though decreases in fertility and egg production have been observed. *Salmonella* Gallinarum causes "Fowl typhoid" in young and particularly adult birds (Shivaprasad, 2000). Clinical symptoms are very similar to those observed during infections with biovar Pullorum. In contrast, infection with *Salmonella* Enteritidis are typically asymptomatic in adults birds, and transovarian transmission of Serotype Enteritidis has also been described (Guard-Petter, 2001). *Salmonella* Enteritidis, as well as serotypes Typhimurium, Kentucky and Heidelberg, are commonly detected among clinically healthy as well as sick chickens and turkeys, indicating a potentially important risk for human health (Hoelzer *et al*., 2011). In contrast to zoonotic *Salmonella* serovars, *Salmonella* Gallinarum do not cause any gastroenteric disease in humans transmitted throughout the food chain. Farmers still experience great losses (due to mortality, morbidity and drop in egg production) caused by host adapted *Salmonella* serovars despite huge amounts spent on vaccination and medication. In early life, *S*. Pullorum causes extremely high mortality of both broilers and commercial laying birds. Similarly, older birds succumb heavily to other serovars of *Salmonella* and it is assumed that *Salmonella* infections of this category of birds are mainly due to *S*. Gallinarum (Mamman *et al*., 2014.)

Poultry salmonellosis related to host adapted serovars remains a major constraint on poultry production in many parts of Nigeria (Mbuko *et al*., 2009).

2.4.2.1 Clinical signs of Avian Salmonellosis and Hematologic changes in Birds

Pullorum disease manifests itself predominantly as an enteric disease of chickens, while fowl typhoid shows signs of septicemic disease. Both biovars can cause septicemic infections, which may be acute or chronic, but unlike *S.* Pullorum, *S.* Gallinarum is capable of producing peracute infection and haemolytic anemia in both young and adults. Other signs include anorexia, weakness, depression, somnolence, loss of appetite, drooping wings, huddling, dehydration and ruffled feathers Labored breathing or gasping, as well as diarrhea and pasting of the vent feathers, may be seen. The droppings can be white and viscous in pullorum disease and watery to mucoid diarrhea in fowl typhoid. Non regenerative anemia seem to be the most common type of anemia described in birds with Salmonellosis showing chronic inflammatory conditions (CFSPH, 2009; Lewandrowski, 2015). Leukocytosis is usually due to heterophilia, Observed granulocytosis which was characterized by myelocytosis and an enhanced rate of the young white blood cells were seen 24 hours following following *S*. Gallinarum endotoxaemia in cockerels (Kokosharov, 2002). Cross contamination in the hatchery is crucial for spreading the disease. Birds can become chronic carriers passing the disease to their offspring through eggs. Horizontal transmission usually takes place orally although sometimes occurs by respiratory route when dry manure is manipulated in the farm and the hen inspires the dust (Terzolo and Chacana, 2016). *Salmonella* is able to pass through the intestinal wall following three alternative routes: (i) *Salmonella* may cross M cells of the Peyer's patches located near the entrance of

caecum, (ii) through diffuse lymphatic tissue of the gut; (iii) alternatively *Salmonella* may enter by the apical pole of intestinal cells and once inside the cytoplasm produces a "*Salmonella* containing vacuole". Inside this vacuole *Salmonella* Gallinarum replicates and the bacterial cells are transported to the basolateral side of the cell in where they are liberated in the lamina propria (Terzolo and Chacana, 2016). The third way of entrance is through a dendritic cell. This dendritic cell emits pseudopods between epithelial cells and capture *Salmonella* that are located in the lumen. In the lamina propria *Salmonella* is engulfed by macrophages. In this sub-epithelial location *Salmonella* causes macrophage apoptosis, process that triggers the inflammation cascade that attracts more phagocytes. Invasion through the brush border and the T junctions of the intercellular space do not cause any damage and therefore goes unnoticed to the immune system. In addition, the lack of flagella of *Salmonella* Gallinarum provides a hiding advantage, as the presences of flagellar proteins (which are highly antigenic) stimulate the immune response (Terzolo and Chacana, 2016). *Salmonella* are different to other intracellular pathogens that multiply free in the cytoplasm, as *Salmonella* induces the formation of intracellular vacuoles. These vacuoles mature in 1 hour and after 3 hours latency *Salmonella* multiplies inside the vacuole. Inside the vacuole *Salmonella* is protected from the action of antibodies, lysozymes and from antibiotics that are capable of intracellular action (Terzolo and Chacana, 2016). Macrophages carry *Salmonella* Gallinarum inside the "Salmonella containing vacuole" and disseminate *Salmonella* Gallinarum systemically. Infected macrophages enter by diapedesis into blood vessels and are dragged by the blood stream to the reticulum endothelial and reproductive tissues (Terzolo and Chacana, 2016). Once *Salmonella* reached the liver following systemic infection, colonisation of the gall bladder and bile ducts of the liver takes place, in where bacteria replicates extracellularly in the lumen and actively invade the gall bladder epithelium.

Afterwards bacteria replicate inside hepatic epithelial cells, into *Salmonella*-containing vacuoles but, at this stage of infection, do not translocate to the lamina propria and mucosa (Terzolo and Chacana, 2016). This intracellular infection leads to a local inflammatory response mediated by heterophils with subsequent tissue damage and epithelial desquamation, leading to a massive release of *Salmonella* cells and hepatic cellular debris into the lumen. A massive release of cells and *Salmonella* clogged the bile ducts and liberates bile into hepatic tissues, which acquire a green colour. At this stage, high numbers of *salmonellas*' are secreted together with the bile into the caudal end of the duodenum by the common bile duct. Only then, *Salmonella* Gallinarum populated the intestine, is excreted in high number and contaminates the environment. The endogenous infection occurs around the fifth day post-infection. This interval of time is called pre-patent period, which is basically the time elapsed since the bird is orally infected until the appearance of Fowl Typhoid symptoms (Terzolo and Chacana, 2016). In typhoidal salmonellosis infections, the invasion and septicaemia occurs immediately after oral invasion and intestinal colonisation occurs at a second stage as result of an endogenous infection, when the bird eliminates *salmonellas* into the intestinal lumen via the common bile duct. In contrast, with paratyphoid infections the intestinal colonization occurs immediately after oral infection and invasion and septicaemia happens at a second stage when *Salmonella* have previously colonised the gut (Terzolo and Chacana, 2016). The events in the liver lead to pathognomonic lesions easily recognized during the necropsy. Adult birds suffering acute Fowl Typhoid usually have a swollen, friable, often bile stained liver due to the destruction of the epithelial cells of the gallbladder and the bile ducts, which leads to a severe stasis of bile. After a fortnight the infection becomes chronic with development of round whitish necrotic foci that are included into the hepatic parenchyma (Terzolo and Chacana, 2016).

2.4.2.2 Immune response against Salmonella Infection

The immune response to *Salmonella* infections is very complicated and involves the interaction of many components of the immune system including the innate and the adaptive immune system (Revolledo, 2012). Although progress has been made in understanding immune responses against *Salmonella* infections, further research is needed to understand the complete roles of humoral and cell-mediated immunity because until now, no consistent pattern has been observed. Pathogenic bacteria have evolved mechanisms to invade the epithelial cell barrier and survive within host tissues. *Salmonella* maintains genes organized within pathogenicity islands that encode virulence factors that allow adherence, invasion and dissemination in the host (Aziz *et al*., 2007). TLR (Toll-like receptors) are cell receptors which recognize structural motifs on pathogens and initiate signaling cascades controlling the development of innate immune response (Chaussé *et al*., 2011). These receptors contribute to host resistance to microbial pathogens and can drive the evolution of virulence mechanisms (Arpaia *et al*., 2011) and can promote adaptive immunity through control of dendritic cell maturation (Iwasaki and Metzhitov, 2004). The consequences of *Salmonella* infection on the expression of the different TLR, and particularly TLR4, have been widely studied (Crhanova *et al*., 2011). Chickens infected with enteric *Salmonella* serovars show high levels of specific antibodies, a T cell response, cytokines and chemokines. Within cell populations, their function can be further discriminated by the presence of cellular determinants, such CD4+ (T helper cells) and CD8+ (T cytotoxic cells), which are associated with helper and cytotoxic functions respectively (Jeurissen *et al*., 2002). The local immune response in the gut has been shown to be more effectively involved in the clearance of *Salmonella* Enteritidis from the gastrointestinal tract than in the systemic response (Desmidt *et al*., 1998). An important role of local cell-mediated immunity in the defense of

chickens against *Salmonella* exposure has been suggested (Berndt and Methner, 2001), describing that modifications of T-cell populations, especially CD8+TcR1+ (γδ) (T-cell receptor-bearing cells) cells in ceca, occur few days after the inoculation of day-old chicks with the serovar Typhimurium. It has been suggested that intestinal SIgA (secretory IgA) responses partially contribute to the later elimination of the *Salmonella* Enteritidis from the gut, and the humoral systemic and local immune responses seem to be related to the cecal colonization (Berthelot-Hérault, 2003). Cell-mediated immunity is responsible for tissue clearance, but how this mechanism could be responsible for intestinal clearance remains unclear (Zhang-Barber *et al*., 1999). The role of T cell responses in the clearance of enteric *Salmonella*e has not been proven. However, in the absence of an essential role for B cells (bursa-derived cells) and with faster clearance of infection as a secondary challenge, the responses are likely to be important evidence for immune memory (Revolledo, 2012).

2.4.2.3 Vaccines against Salmonella and Immune Mechanisms of Protection

In chickens, vaccines should prevent intestinal and cecal colonization resulting in diminished fecal shedding, and should be effective against systemic infection preventing vertical transmission and egg contamination. Vaccinations with either inactivated or live products have been applied for reducing the susceptibility of poultry to *Salmonella* infections (Gast, 2007). Both inactivated and live vaccines are used to protect against *Salmonella* challenge, however, killed products increased humoral immunity and reduced *Salmonella* prevalence but did not significantly decrease *Salmonella* in the farm environment (Berghaus *et al*., 2011). Vaccines based on dead *Salmonella* bacteria have been used to protect poultry and its progeny against field

challenges. Inactivation is usually achieved by either heating or the use of formalin and an adjuvant. However, there are two problems with these products, they fail to elicit a cell-mediated immune response, which is considered the most important for clearing the intracellular pathogen (Lax *et al*., 1995), and they do not stimulate secretory IgA (SIgA) responses at mucosal surfaces, which is the key for protection against intestinal colonization (Cerquetti and Gherardi, 2000). Subunits vaccines, like outer membrane proteins, porins, and ribosomal fractions have been tested to prevent *Salmonella* infections. The efficacy of these products is not good when administered without an adjuvant or a delivery system (Gamazo and Irache, 2007). Live and attenuated vaccines have been used worldwide, and their efficacy has been demonstrated in challenge trials (Papezova *et al*., 2008; Atterbury *et al*., 2010). The principle behind the use of a live attenuated vaccine should be to reduce the bacterial virulence while maintaining its immunogenicity. Attenuated strains use in live vaccines include semi-rough strains, such as *Salmonella* Gallinarum 9R strain (Kwon and Cho, 2011), auxotrophic and metabolic drift mutants. Others attenuated by biological gene-deletion techniques include *aroA* mutants, *crp* mutants, this genetically modified organisms are permitted in the USA, and other countries, except Europe. It is important to evaluate the behavior of the attenuated bacteria strains in the environment, focusing on the attenuated strain obtained by gene deletions, because they can acquire genes from other microorganisms and recover their virulence. Vaccines should establish a long lasting immunity by manipulating the cytokine milieu to induce the appropriate effector mechanisms for each particular pathogen and by creating a large pool of long lived memory cells (Chabalgoity *et al*., 2007). Vaccines used in poultry against *Salmonella* infections have been effective, but they were empirically designed, and are not based on detailed information about the immune responses of protection because efficacy is frequently evaluated in challenge trials.

The route of vaccination is important in influencing immune responses at the initial site of pathogen invasion where protection is more effective (Belyakov and Ahlers, 2009). Mucosal dendritic cells play an important role in the induction and maintenance of protective immunity against pathogens like *Salmonella*. Dendritic cells are responsible for antigen presentation following mucosal vaccination and systemic immunization have a limited effect on the delivery of antigen to mucosal dendritic cells (Coombes and Powrie, 2008). An important difference must be established in *Salmonella* attenuated vaccines regarding the immune response and the administration route. Parenteral vaccines stimulate a strong humoral response, while oral live attenuated vaccines generate both mucosal and systemic immunity (Bouvet *et al*., 2002). There is a fundamental difference between inactivated and attenuated live vaccines. Live attenuated *Salmonella* vaccines stimulate the secretion of mediators (cytokines, interleukin and interferon) inducing a T helper 1 response and cell-mediated immunity; in different animal species and in poultry this type of immune response is not induced by inactivated products. Additionally, CD8+ T cells play a crucial role in immune protection against intracellular pathogens, such as *Salmonella*, and these cells (CD8+ T) response are exclusive after vaccination using live attenuated vaccines. Attenuated live bacteria vaccines applied by oral route are excellent tool for mucosal immunization (Husseiny and Hensel, 2005), because mucosal surfaces are the first interface between *Salmonella* and the host. The first step to initiating an immune response in the gut surface by oral vaccines is based on the signals sent by receptors for pathogen-associated molecular patterns (PAMP) via pathogen recognition receptors (PRR), such as TLR. TLR in chickens are very similar to those in mammals; however, some differences in recognition patterns related to TLR5, which recognize flagellin are observed in host specific *Salmonella* and non-host-specific *Salmonella* strains (Revolledo, 2012). In response to flagellin lamina propria

dendritic cell, the differentiation of naïve B cells into IgA plasma cells occurs via a mechanism that is independent of the gut associated lymphoid tissue (GALT) (Revolledo, 2012). Recognition of *S.* Typhimurium is largely mediated by TLR2, TLR4 and TLR5 (Uematsu *et al*., 2006) and TLR9 is involved in the regulation of the replication of the bacterium. Lipopolysaccharide (LPS) of *Salmonella* is recognized by TLR4 expressed at the surface of the immune cells and in the cytoplasm of intestinal epithelial cells (Crhanova *et al*., 2011). The second step in the immune response is related to the ability of the antigen to cross the epithelial barrier and to be presented to antigen presenting cells, especially dendritic cells. Mucosal dendritic cells play a central role in the induction of protective immunity against invasive pathogens. Unique dendritic cell subsets are responsible for antigen presentation following mucosal vaccination (Belyakov and Ahlers, 2009). To achieve protection, it is important that memory cells are generated in enough numbers and persist as a functional long-lived population (Chabalgoity *et al*., 2007). Some authors indicated that it would seem crucial that the vaccine strains retain capacity of invasiveness in order to stimulate sufficient immunity to be protective (Barrow, 2007; Van Immerseel, 2016), however, the immune response in the intestinal mucosa has revealed new possibilities because oral antigens induce effector and memory cells that express certain receptors only on lymphocytes of intestinal mucosa (Aziz *et al*., 2007).

2.5 *Salmonella* Kentucky

Salmonella enterica subsp. *enterica* serovar Kentucky (antigenic formula 8, 20: i: z6) is a serovar of the O: 8 (C2-C3) serogroup. *Salmonella enterica* subsp. *enterica* serovar Kentucky is frequently isolated from healthy poultry and dairy cows and is occasionally isolated from people

with clinical disease (Haley *et al*., 2016; Switt, 2017). *Salmonella enterica* serotype Kentucky (*S.* Kentucky) has been closely associated with poultry since it was first isolated in 1937 from a chick in the United States of America (USA) (Crump *et al*., 2015).

2.5.1 Geographical distribution of *Salmonella* Kentucky

S. Kentucky is distributed worldwide. It is commonly found in animals (specifically cattle and poultry) and in meat. In Europe, U. S., Asia and Africa, this serovar is also frequently isolated from humans with symptoms consistent with Salmonellosis (Haley *et al*., 2016; Switt, 2017).

2.5.2 Relevant genetic characteristics of *Salmonella* Kentucky

Whole genome sequences for two strains of *S.* Kentucky, one from a human (str. CDC 191) and one from chicken breast (str. CVM29188), have been deposited at GenBank as of October, 2013. Genomic characteristics of the sequenced *S.* Kentucky include (i) a genome size ranging from 4.7-5.09 Mb, (ii) a mol G+C% ranging from 51.9-52.2, and (iii) 4,735 to 5,295 predicted genes. *Salmonella* Kentucky str. CVM29188 is a multidrug resistant strain that contains three plasmids of 46, 101, and 146 kb. The two large plasmids are conjugative plasmids that carry antimicrobial resistance genes. The largest plasmid of 146 kb shows the same backbone and virulence genes as virulence plasmids of avian pathogenic *E. coli* (APEC). Partial sequences of a region in *Salmonella* Kentucky strain SRC73 are deposited at GenBank. This strain, from Indian spices, is a multidrug resistant strain that contains the resistance genes inserted in the chromosome in a genomic island (*Salmonella* Genomic Island 1 variant SGI1-K) (Haley *et al*., 2016).

Phylogenetic and pan-genomic analyses that included completely sequenced *S.* Kentucky and other *Salmonella* serovars concluded that *S.* Kentucky (i) belongs to clade A, according to Den-Baker *et al*. (2011) classification, (ii) appears to have several evolutionary origins, and (iii) contains 127 gene families unique to this serovar (Switt, 2017).

2.6 Virulent Factors of *Salmonella* spp

The virulence seen in *salmonella* species is associated with a combination of chromosomal and plasmid factors (Oliveira *et al*., 2003), and many studies have identified genes that encodes these factors. Pathogenesis of salmonellosis depends upon a large number of factors controlled by an array of genes that synergize into the actual virulence of *Salmonella* (Murugka *et al.,* 2003). Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Some genes are known to be involved in adhesion and invasion *viz.*, *sef*1, *pef*2, *spv*3 or *inv*4 *(*Murugka *et al.,* 2003). Others are associated with the survival in the host system- *mgt*C5 or in the actual manifestation of pathogenic processes *viz.*, *sop*6, *stn*7, *pip* A, *pip* B, *pip* D8. (Murugka *et al.,* 2003). A number of virulence factors of *Salmonella* have been identified e.g Virulence-plasmids, toxins, fimbriae, adhesion, invasion, efflux pumps etc (Alphons and Jaap, 2005). More than 300 genes have been annotated as regulatory genes in *Salmonella* and of those, 14 regulators including *SpvR*, *FruR*, *IHF*, *PhoP/PhoQ*, *SsrA/SsrB*, *SlyA*, *Hnr*, *RpoE*, *SmpB*, *CsrA*, *RpoS*, *CRP*, *OmpR/EnvZ*, and *Hfq* are required for virulence regulation during systemic infection in an acute mouse infection model (Yoon *et al*., 2009; Yoon *et al*., 2011). Many of these virulence factors (adhesion, invasion, and toxin genes etc) in *Salmonella* are clustered in certain areas of the chromosome known as "*Salmonella* pathogenicity islands" (SPI) (Santos *et*

al., 2003). SPI are characterized by a base composition different from the core genome and are often associated with tRNA genes and mobile genetic elements, like IS (Insertion sequence) elements, transposons or phage genes (Schmidt and Hensel, 2004). Until recently, five SPIs (termed SPI-1 to SPI-5) have been identified on the *Salmonella* chromosome. Now15 SPIs have been identified in *S.* Typhi (Parkhill *et al*., 2001; Vernikos and Parkhill, 2006). SPI-1 has been identified as a 40 kb DNA region encoding a Type III secretion system (T3SS) involved in host invasion and intracellular proliferation (Hansen-Wester and Hensel, 2001). This type III system transports bacterial proteins like the actin binding proteins *SptP* and *SopE* into the cytosol of the target cell that leads to an uptake of the bacterium by the cell (Hayward and Koronakis, 2002). The 40 kb SPI-2 locus encodes a second type III secretion system and is required for bacterial survival in both epithelial cells and macrophages (Kuhle and Hensel, 2004). SPI-2 is required for intracellular pathogenesis and has a crucial role for systemic *Salmonella enterica* infections (Hansen-Wester and Hensel, 2001). SPI-3 harbors 10 open reading frames amongst which the *mgtC* gene. This *Salmonella* specific gene is required for growth in an Mg2+ limiting environment like assumed in phagosomes (Blanc-Potard and Groisman, 1997). SPI-4 is needed for intra-macrophage survival and is likely to carry a type I secretion system involved in toxin secretion (Wong *et al.,* 1998). SPI-5 revealed the presence of six genes including the *SopB* gene, a gene encoding an effector protein (Alphons and Jaap-van Dijk, 2005). Type III protein secretion systems (T3SS) although termed secretion systems the biological function of T3SS is the translocation of proteins (e.g. *SptP* and *SopE)* from the bacterial cytoplasm into the host cell, thus functioning as 'molecular syringes' (Hueck, 1998). T3SS is an ATP-dependent system and are restricted to Gram-negative bacteria. They perform distinct functions ranging from antiphagocytic and cytotoxic effects on host cells (*Ysc/Yop* system of *Yersinia* spp.), invasion of

host cells (*S. enterica* SPI-1 system), intracellular pathogenesis (*S. enterica* SPI-2 system). The T3SS is a complex of proteins that allows for the transfer of virulence factors directly into the host cells and is associated with at least 20–30 structural and regulatory proteins involved in cellular invasion. (Marlovits *et al*., 2004; Gala'n and Wolf-Watz, 2006; Marlovits and Stebbins, 2010). Many of the genes encoded on SPI-2 encode for the structure of secretion system apparatus (ssaG through *ssa*U), secretion system effector (*sse*ABCDEF), secretion system chaperones (*ssc*AB), and secretion system regulatory (*ssr*AB) genes required for a functional T3SS (Hensel, 2000). Certain *Salmonella* serovars belonging to subspecies I carry a large, low copy-number plasmid that contains virulence genes. Virulence plasmids are required to trigger systemic disease (Rotger and Casadesús, 1999). *Salmonella* virulence plasmids (*spv)* are heterogeneous in size (50–90 kb), but all share a 7.8 kb region. *spv* are required for bacterial multiplication in the reticuloendothelial system (Rotger and Casadesús, 1999) and this *spv* 7.8 kb region is necessary to confer the virulent phenotype (Alphons and Jaap-van Dijk, 2005). This locus harbors five genes designated *spv* RABCD (Alphons and Jaap-van Dijk, 2005). The product of the *spvR* is a positive regulatory protein essential for the expression of the other *spv* genes (Alphons and Jaap-van Dijk, 2005). The *spvB* product is an ADP ribosylates actin, which function in destabilizing the cytoskeleton of the eukaryotic cell (Lesnick, 2001). Proteins encoded by the virulence plasmid genes rck (resistance to complement killing) play a role in antiphagocytic process (Haneda, 2001). Other loci of the plasmid, such as the fimbrial operon *pef*, (biosynthesis of fimbriae) and the enigmatic *rck* and *rsk* loci may play a role in other stages of the infection process and in serum resistance (Gulig, 1993; Guiney, 1994). *S.* Kentucky was reported to habour a *colV* virulent plasmid which significantly increased its ability to colonize the chicken cecum and cause extra-intestinal disease (Johnson *et al*., 2010). *rsk* (regulation of

serum killing) might be involved in resistance of *Salmonella* spp. to the bacteriolytic activity of serum (Alphons and Jaap-van Dijk, 2005). *Salmonella* produces both endotoxins and exotoxins. The exotoxins can be subdivided in two types; the cytotoxins and the enterotoxins. Cytotoxins (also called verotoxins; referring to a cytotoxic assay in Vero cells are defined by their ability to kill mammalian cells (Alphons and Jaap-van Dijk, 2005). Fimbriae (also called pili) are filamentous surface structures that are 2–8 nm wide and 0.5–10 nm long mainly composed of helically arranged repeated proteins called fimbrins (Alphons and Jaap-van Dijk, 2005). The 8–11 genes encoding for proteins involved in biosynthesis, structure and assembly are generally clustered in a 7–9 kb large operon (Alphons and Jaap-van Dijk, 2005). The *agf* operon for example is aggressive fimbriae promotes the initial interaction of the bacteria with the intestine of the host and stimulate bacterial self-aggregation, resulting in higher rates of survival (Collinson *et al*., 1993). Three pathways for the assembly of fimbriae in members of the family of *Enterobacteriaceae* have been described namely the Nucleator dependent pathway, the Assembly pathway for so called type IV fimbriae and the Chaperone–usher-dependent pathway (Townsend *et al*., 2001). The *agf* operon (an operon of the nucleator-dependent pathway) is an aggressive fimbriae that promotes the initial interaction of the bacteria with the intestine of the host and stimulate bacterial self-aggregation, resulting in higher rates of survival (Collinson *et al*., 1993). This *agf* operon, showed sequence involved in the synthesis of thin aggregative fimbriae called Curli (Alphons and Jaap-van Dijk, 2005). Curli production positively affects biofilm formation. Homologs to the agf operon are found in all six subspecies of S. enterica and in *S.* bongori (Alphons and Jaap-van Dijk, 2005). In *Salmonella* Enteritidis fimbriae encoded by the *agf* operon are designated SEF17 fimbriae or thin aggregative fimbriae (Tafi) (White *et al*., 2003). A biofilm can be broadly defined as a microbially derived sessile community

characterized by cells that are irreversibly attached to a substratum or interface or to each other, and are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Kuchma and O'Toole, 2000; Donlan and Costerton, 2002; Lazazzera, 2005; Shemesh *et al*., 2007). Interestingly, it has been observed that the resistance of biofilm cells to antimicrobials is significantly increased compared with what is normally seen with the same cells being planktonic (Mah and O'Toole, 2001; Gilbert *et al*., 2002). Thus, it is believed that biofilm formation enhances the capacity of pathogenic *Salmonella* bacteria to survive stresses that are commonly encountered both within food processing, as well as during host infection (Efstathios *et al*., 2012). Several reports have demonstrated the ability of *Salmonella* to form biofilms on biotic and abiotic surfaces outside the host such as stainless steel, plastic and cement (Hood and Zottola, 1997; Giaouris *et al*., 2005; Giaouris and Nychas, 2006; Chia *et al*., 2009; Marin *et al*., 2009). *Salmonella* Typhimurium *BarA* and *SirA* are two component systems that are important for biofilm formation and virulence in many c-Proteobacteria. *SirA* activates the *csrB* and *csrC* carbon storage regulatory RNAs and the virulence gene regulators *hilA* and *hilC.* The regulatory RNAs antagonize the activity of the *CsrA* protein, allowing translation of those same virulence genes, and inhibiting the translation of flagellar genes, which are inhibitory to biofilm formation (Teplitski *et al*., 2006). *S. enterica* serovar Kentucky has shown an increase in several countries with the concurrent emergence of multidrug-resistant isolates. A total of 57 *Salmonella* Kentucky strains were isolated from different sources during the period 2005 to 2008 in Tunisia. Majority of these strains tested were able to form a biofilm, especially for environmental and animal derived isolates. Therefore, the biofilm seems to comprise a normal and favourable capability in the life of *Salmonella* Kentucky in the environment (Turki *et al*., 2012). Multidrug

resistance (MDR) refers to the capability of bacterial pathogens to withstand lethal doses of structurally diverse drugs which are capable of eradicating non-resistant strains (Jingjing *et al*., 2014). Efflux pumps are found in almost all bacterial species and genes encoding this class of proteins can be located on chromosomes or plasmids (Piddock 2006; Poole 2007). Drug extrusion by the multidrug efflux pumps serves as an important mechanism of MDR. Multidrug efflux pumps have important physiological functions, including transport of drugs, bile salts, toxins, and environmental compounds (Nikaido *et al*., 2008). Efflux pumps can expel a broad range of antibiotics due to their poly-substrate specificity and also drive the acquisition of additional resistance mechanisms by lowering intracellular antibiotic concentration thereby decreasing cellular drug accumulation and promoting mutation accumulation (Nikaido *et al*., 2008; Jingjing *et al*., 2014). According to their composition, number of transmembrane spanning regions, energy sources and substrates, bacterial efflux pumps are classified into five (Handzlik *et al*., 2013).

1. The Resistance- nodulation-division (RND) superfamily.
2. The Major facilitator superfamily (MFS).
3. The ATP (adenosinetriphosphate)-binding cassette (ABC) superfamily.
4. The Small multidrug resistance (SMR) family (a member of the much larger drug/metabolite transporter (DMT) superfamily).
5. The multidrug and toxic compound extrusion (MATE) superfamily (Putman *et al*., 2000; Poole, 2007).

The RND Superfamily is only found in Gram-negative bacteria while efflux systems of the other four families (MFS, ABC, SMR and MATE) are widely distributed in both Gram-positive and negative bacteria (Handzlik *et al*., 2013). RND type efflux pumps are multiple-component

systems containing an inner membrane transporter and also an outer membrane channel and a periplasmic adaptor protein (Li and Nikaido, 2004). Piddock, (2006) reported that mutation in *acrR* contributes to over expression of *acrAB* in *Salmonella* and increases resistance to multiple drugs. RND family pumps have been found to be associated extensively with clinically significant antibiotic resistance *Salmonella* Typhimurium. The *AcrAB-TolC* efflux system in *S.* Typhimurium was also found to be able to expel different classes of antimicrobial agents such as quinolones, chloramphenicol, tetracycline and nalidixic acid (Poole, 2007). *S. enterica* serovar Typhimurium phage type DT204 has been reported to result from multiple target gene mutations and active efflux by the *AcrAB-TolC* multidrug efflux pump (Nikaido *et al*., 2008).

2.7 Mutation and Mechanisms of Mutagenesis

Mutagens induce mutations by at least three different mechanisms. They can replace a base in the DNA, alter a base so that it specifically mispairs with another base, or damage a base so that it can no longer pair with any base under normal conditions (Griffiths *et al*., 2000). Some chemical compounds are sufficiently similar to the normal nitrogen bases of DNA that they occasionally are incorporated into DNA in place of normal bases; such compounds are called base analogs. Once in place, these analogs have pairing properties unlike those of the normal bases; thus, they can produce mutations by causing incorrect nucleotides to be inserted opposite them in replication. The original base analog exists in only a single strand, but it can cause a nucleotide-pair substitution that is replicated in all DNA copies descended from the original strand (Griffiths *et al*., 2000).

2.7.1 5-Bromouracil

5-bromouracil (5-BU) is an analog of thymine that has bromine at the C-5 position in place of the methyl (CH3) group found in thymine. This change does not affect the atoms that take part in hydrogen bonding in base pairing, but the presence of the bromine significantly alters the distribution of electrons in the base. 5-BU can frequently change to either the enol form or an ionized form; the latter pairs in vivo with guanine. Thus, the nature of the pair formed in replication will depend on the form of 5-BU at the moment of pairing. 5-BU causes transitions almost exclusively, been an analog of thymine, it can be incorporated into DNA as a base (Griffiths *et al*., 2000). In its normal Keto state, it mimics the pairing behaviour of thymine that it replaces pairing with adenine. The presence of the bromine atom however, causes a relatively frequent redistribution of electrons, so that 5-BU can spend part of its existence in a rare ionized form. In this state, it pairs with guanine, mimicking the behaviour of cytosine and thus inducing mutations in replication (Griffiths *et al*., 2000).

2.8 Diagnosis of *Salmonella* spp

Diagnosis is based on the isolation of the organism using a variety of techniques that may include pre-enrichment to resuscitate sub-lethally damaged Salmonellae, enrichment media that contain inhibitory substance to suppress competing organism and selective plating agars to differentiate Salmonellae from other enterobacteria. Isolation could be either from tissues collected aseptically at necropsy or from faeces, rectal swabs or environmental samples, food products and feedstuffs. When infection of the reproductive organs, abortion or conceptus

occurs, it is necessary to culture foetal stomach contents, placenta and vaginal swabs and in the case of poultry, embryonated eggs (OIE, 2010).

2.9 Control of *Salmonella* spp

Effective control of food-borne pathogens, including *Salmonella*, is a major challenge to the poultry industry. Control of *Salmonella* infection in chicken focuses partly on farm management, although vaccination is sometimes used in Europe to control *S.* Enteritidis infection (Atterbury *et al*., 2009). For vaccine prevention, both killed virulent and live attenuated Salmonella strains are used (Holt *et al*., 2003; Atterbury *et al*., 2009). Although killed and attenuated *Salmonella* vaccines reduce intestinal colonization, and egg and meat contamination, the level of protection is variable, dependent in part on the serovars involved (McHan *et al*., 1991; Jiang *et al*., 2010). Live attenuated *Salmonella* vaccines have been shown to induce both humoral and cell-mediated immune responses similar to those observed after natural infections (Sasai *et al*., 1997; Mastroeni *et al*., 2001), and have the benefits of ease of administration and low cost. *Salmonella* have promise as delivery vectors for control of bacterial and viral infections in chickens (Babu *et al*., 2003; Konjufca *et al*., 2006; Kong *et al*., 2008; Kulkarni *et al*., 2010; Medina *et al*., 2001). In recent years, improvements of attenuated *Salmonella* as vaccine vehicles have included regulated delayed attenuation (Curtiss *et al*., 1991) and regulated antigen synthesis (Wang *et al*., 2010) such that strains express protective antigens at high levels and stimulate strong and lasting immune responses without producing tissue damage, while improving their immunogenicity and safety (Kong *et al*., 2010; Wang *et al*., 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bacteria Isolate

Field isolate of *S.* Kentucky was obtained from Fagbamila *et al*. (2017) of the bacterial research division of National Veterinary Research Institute Vom, which was identified and serotyped (somatic O antigen factors 8,20, flagellar H antigen i:z6) according to the Kauffmann-White Scheme at the OIE *Salmonella* reference laboratory, Padova, Italy (Istituto Zoopofilattico Sperimentale delle Venezie) ISO 6579:2002.

3.2 Experimental Birds

A total of 70 day old Zartech broiler chicks were obtained from Ibadan, Nigeria and housed in the animal experimental unit of the National Veterinary Research Institute Vom, Nigeria. Cloacal swabs were taken and pre-enriched in buffered peptone water (Oxoid, UK) (Appendix VIII) in a 1:10 sample to broth ratio at 37 °C for 18 hours. 0.1 mL of the pre-enriched broth was inoculated into Rappaport-Vassiliadis (RV) (Oxoid, UK) incubated for 18 hours at 37 °C. Approximately 100 µL (3 drops) were plated onto Xylose lysine deoxycholate (XLD) agar (Oxoid, UK). The plate was incubated at 37 °C for 24 hours to ascertain their *Salmonella* spp status. The procedures used for animal care, housing and experimentation were in accordance with the ABU Committee on Animal Use and Care (ABUCAUC).

3.3 Vaccine Preparation

3.3.1 Attenuation of *Salmonella* Kentucky using 5-Bromouracil

Stock culture of field isolate of *S.* Kentucky were produced by picking a discrete colony of *S.* Kentucky cultured on Xylose lysine deoxycholate (XLD) agar (Oxoid, UK) plates and inoculated into 10 mL Nutrient broth (NB) (Oxoid, UK) (Appendix VI), incubated at 37 °C for 24 hours. The NB culture containing *S.* Kentucky were further inoculated separately into five Nutrient broths (NB) (Oxoid, UK) (1 mL stock culture to 10 mL NB) and incubated at 37 °C in a graduated 50 mL bottle with for 18 hours. The cultures were harvested by centrifugation at 10,000 rpm for 10 minutes. The sediments were washed and re-suspended in 10 mL of Citrate buffer (Appendix IX) of pH 5.5. 1 mL each of the culture was taken for colony count in (CFU/mL) by a 10 fold serial dilution in 0.85 % Normal saline in 10 test tubes; 0.05 mL aliquots dilution each of last five dilutions was spread onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK). 5-bromouracil (Sigma-Aldrich, Germany) at the following final concentrations 400 µg/mL (0.4 mg in 1 mL citrate buffer), 600 µg/mL (0.6 mg in 1 mL citrate buffer), 800 µg/mL (0.8 mg in 1 mL citrate buffer), 1,000 µg/mL (1.0 mg in 1 mL citrate buffer) and 1,200 µg/mL (1.2 mg in 1 mL citrate buffer) were added to the suspensions of the bacteria (Hertman *et al*., 1978). The bacterial suspensions containing different concentrations of 5-bromouracil were incubated in a water bath for 30 minutes at 37 °C. After 30 minutes 0.5 mL each of the culture was further taken for viability and colony count by 10 fold serial dilution of the bacteria in 0.85 % Normal saline and subsequent plating on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK). Culture was then re-centrifuged at 10,000 rpm for 10 minutes and sediment washed twice with 10 mL phosphate buffer (Appendix IX) of pH 7.2. The washed sediment containing the treated bacteria was re-suspended in 10 mL of fresh NB (Oxoid, UK) and incubated for 18 hours

at 37 °C in flask with reciprocal shaking. At the above concentrations of 5-bromouracil, the percentage live bacterial count was determined. Only cultures with less than 20 % bacterial live count after exposure to 5-bromouracil were taken for further isolation. Bacterial colonies surviving 5-bromouracil treatment were picked up by sterile Pasteur loops, purified by twice streaking on XLD agar (Oxoid, United Kingdom) plate surfaces and re-isolation of single colonies following incubation at 37 °C for 24 hours (Hertman *et al*., 1978).

3.3.2 Virulence test

Infective dose of 5-BU attenuated *Salmonella* Kentucky and non-attenuated *Salmonella* Kentucky stock was prepared to an approximate stock dilution of 1 x 10^9 CFU/mL bacteria suspension in sterile Nutrient broth (Oxoid, UK). Five 14-day-old broiler chicks per group were infected by oral administration of 1 mL of broth culture and observed for clinical signs such as profuse white diarrhoea, depression somnolence and mortality. Gross lesions looked out for were swollen liver and necrotic foci in the liver (Osman *et al*., 2010; Nourhan *et al*., 2014). Reisolation and quantification of *Salmonella* from the liver was undertaken. 1 g of liver sample was homogenized in 10 mL of 2 % buffered peptone water (Oxoid, UK). 1 mL of the pre-enriched broth was inoculated into Rappaport-Vassiliadis (RV) (Oxoid, UK) incubated for 18 hours at 37 °C. Colony count by 10 fold serial dilution of the bacteria in 0.85 % Normal saline and subsequent plating on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK) undertaken. The number in CFU/mL transformed to Log_{10} and percentage re-isolation of *Salmonella* Kentucky from liver on XLD (Oxoid, UK) was calculated (Borsoi *et al*., 2011; OIE, 2012; Baxter, 2015).

Percentage (%) Reisolation = $Log_{10}N/9.0 \times 100$

$Log_{10}N = Log_{10}$ CFU/mL, following Reisolation, $Log_{10}(1 \times 10^9$ CFU/mL) = 9.0

3.3.3 Preparation of *Salmonella* Kentucky Concentrate

Nutrient agar slants were prepared in Roux flask (28 g of Nutrient agar powder (Oxoid, UK) was suspended in 1 litre of distilled water. Suspension was brought to boil to dissolve completely. 250 mL was dispensed into each Roux flask and sterilized by autoclaving at 121 °C for 15 minutes. Nutrient agar in Roux flasks were slanted on bench in sterile room at 25 °C to solidify). Aseptically, 10 mL of stock culture of 5-bromouracil attenuated and non attenuated *S.* Kentucky was inoculated unto Nutrient agar (Oxoid, UK) slant in Roux flasks and incubated at 37 °C for 48 hours separately. The colonies were harvested with sterile normal saline with glass beads by gentle rocking. Purity was carried out on each roux flask culture by streaking unto Blood agar (BA) plates, plates were incubated at 37 °C for 24 hours while Roux flask cultured stored at 4 °C. 5-bromouracil attenuated and non-attenuated cultures were pooled separately and bacterial suspension adjusted to contain 10^7 CFU/mL using colony count technique (OIE, 2008; Baxter, 2015) for vaccine preparation. Purity of the pooled cultured was checked by plating approximately 100 µL (3 drops) on XLD (Oxoid, UK) and Blood Agar plates (OIE, 2008).

3.3.4 Preparation of Stabilizers

Fifteen grams of gelatin (Oxoid, UK) was dissolved in 100 mL of deionized water and sterilized by autoclaving at 121 °C for 15 minutes. 50 g Lactalbumin hydrolysate (LAH) (Oxoid, UK), 100

g sucrose (Sigma-Aldrich, Germany), 1.16 g potassium dihydrogen orthophosphate (KH_2PO_4) (J.T. Baker, USA) and 7.66 g 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 8.5 g 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 unto BA plates and incubating at 37 °C for 24 hours (OIE, 2008).

3.3.5 Live attenuated Vaccine mixture

Aseptically, the 5-bromouracil attenuated *Salmonella* Kentucky concentrate at approximately 10^7 CFU/mL bacterial suspensions was mixed with stabilizers at a ratio of 5:4:1 (50 % *S.* Kentucky concentrate, 40 % LAH, 10 % Gelatin) thoroughly. The vaccine was dispensed into sterile vials at 2.5 mL and lyophilized at National Veterinary Research Institute Vom where production of the experimental vaccine was undertaken (OIE, 2008).

3.3.6 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, 18 mL of formaldehyde solution 37 % stabilized (Sigma-Aldrich, Germany) was added and mixed thoroughly before plating unto blood agar plate to ascertain for sterility (inactivation) by incubating the Blood agar (BA) plate at 37 °C for 24 hours. Following confirmation of bacterial inactivation, Glycerin saponin saline (GSS) was prepared by mixing 1000 mL of glycerin (Schutz, Indonesia) with 100

g of saponin (Sigma-Aldrich, Germany) and 8.5 g of NaCl (Oxoid, UK) in 1000 mL of deionized water; autoclaved at 121°C for 15 mins in graduated 5000 mL Schott-Duran bottle and added to the killed bacteria suspension at a ratio of 8:2 (8 parts of inactivated bacteria suspension: 2 parts of GSS) and vaccine dispensed into sterile 200 mL bottle (OIE, 2008).

3.4 Quality Control on the Prepared Vaccine

3.4.1 Purity Testing

Five vials of the lyophilized live attenuated vaccine was sampled randomly and reconstituted with 2.5 mL sterile normal saline. Using a Pasteur wire loop, purity check was carried out by streaking each vial separately on paired BA plates and Sabouraud Dextrose agar (SDA) (Appendix VII) 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, SDA plates were incubated at 25°C for seven days. Colony count was undertaken by a 10 fold serial dilution in normal saline and aliquot (0.05 mL) of the last 5 dilutions were cultured on BA plates in duplicate pairs, incubated at 37 °C, for 24 hours. Sterility (inactivation) check was carried out on the killed vaccine 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 (OIE, 2008).

3.5 Experimental Design

Three groups, each with twenty broiler day old chicks were reared in a deep litter system in separate pens;

1. Group 1 received 0.5 mL of the prepared live attenuated *S.* Kentucky vaccine subcutaneously at day 11 of age and a repeat at day 25 of age with the same dose and route of administration.
2. Group 2 received 1.0 mL of the prepared inactivated *S.* Kentucky vaccine subcutaneously at day 11 of age and a repeat at day 25 of age with the same dose and route of administration.
3. Group 3 served as the control (unvaccinated); they were sham inoculated with 0.5 mL sterile normal saline (NS) subcutaneously at day 11 of age and a repeat at day 25 of age with the same dose and route of administration (OIE, 2008).

3.5.1 Challenge Test

The three groups were challenged on day 32 of age by oral administration of 1 mL each of *S.* Kentucky field isolate in nutrient broth containing approximately 5 x 10^7 CFU/mL bacteria suspension (OIE, 2012).

3.5.2 Potency Test

Seroconversion of the experimental chickens to *S.* Kentucky was measured in serum using Chicken IgY Enzyme Linked Immunosorbent Assay (ELISA) kit (Wuhan Fine Biotech Co., Ltd)

according to the manufacturer's directive (Appendix X) (1 µL of serum sample was diluted in 99 µL of Standard Dilution Buffer) and quantification of titre via absorbance at 450 nm wavelength in an ELISA machine (RT-2100C; Rayto Microplate Reader). Serum samples were collected at day 7, day 14, day 21, day 28, day 35, day 42, day 49 and day 54 of age. All serum samples were stored at – 20 °C and ELISA's was run at one time to minimize variability.

3.5.3 Faecal Shedding

Quantitative *Salmonella* Kentucky shed in faeces was 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 isolate. Cloacal Sample was then taken on days 35, 42, 49 and 54. Sample swab were used to transfer 3 g to 5 g faecal sample to a stomacher bag and sample diluted to contain 1 g to 10 mL of 0.85% normal saline (NS). Further 10 fold serial dilution of the sample in 0.85% normal saline in 10 test tubes was carried out and 0.05 mL aliquots of each of the first five dilutions was spread onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK). After 18 to 24 hours of incubation at 37 °C, *Salmonella* colonies (black), on each plate were counted (Gast *et al*., 1993). The prevalence and percentage vaccinal efficacy was calculated using the method of Thrusfield, (2007). Formula;

% vaccinal efficacy = C – T / C x 100, where

C = prevalence unvaccinated animals (unvaccinated animals are defined as 'exposed' to the risk factor); T = prevalence of vaccinated animals.

3.6 Data Analysed

All data obtained from the study were entered into Microsoft excel and analysed with the aid of GraphPad Prism (Version 5.01, Graphpad software Inc.). P values < 0.05 were 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 Killed 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.

CHAPTER FOUR

RESULTS

4.1 Growth of *Salmonella* Kentucky on Xylose Lysine Deoxycholate (XLD) agar

Salmonella Kentucky colonies on XLD (Oxoid, UK) agar appeared as a translucent, circular, raised and smooth with black centres. It has the capacity to ferment Xylose to produce acid and metabolize thiosulfate to produce hydrogen sulfide, which forms colonies with a black centre (Plate I).

4.2 Result of percentage live bacterial count following Attenuation of *Salmonella* Kentucky using 5-Bromouracil

Concentrations of 5-bromouracil at 400 μg/mL, 600 μg/mL 800 μg/mL, 1000 μg/mL and 1200 μg/mL, caused a decrease in live bacterial count in CFU/mL from an initial 1.6×10^{12} CFU/mL (equivalent of 12.204 in Log to base 10) of *S.* Kentucky to 3.40×10^{5} CFU/mL (equivalent of 5.53 in Log to base 10), 2.0×10^{3} CFU/mL (equivalent of 3.30 in Log to base 10), 3.0×10^{2} CFU/mL (equivalent of 2.48 in Log to base 10), 2.00×10^{2} CFU/mL (equivalent of 2.30 in Log to base 10) and 0 CFU/mL (equivalent of 0.00 in Log to base 10) respectively representing a percentage live bacterial count equivalent to 45.31 %, 27.04 %, 20.32 %, 18.85 % and 0 % respectively (Table 4.1).

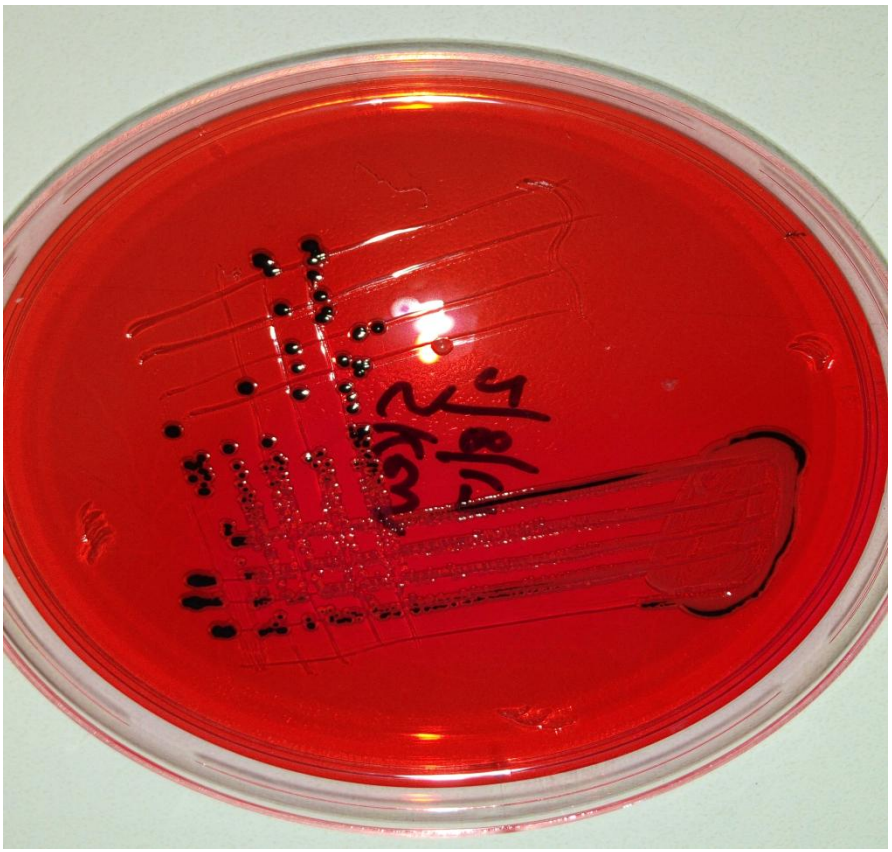


Plate I: Typical translucent colonies with black centres of *Salmonella* Kentucky on XLD (Oxoid, UK)

Table 4.1: Percentage live bacterial count of *Salmonella* Kentucky using 5-bromouracil

5-bromouracil	**CFU/mL (N)**	**$log_{10}N$**	**% live bacterial count ($log_{10}N/12.204 \times 100$)**
400 μg/mL	3.40×10^5	5.53	45.31
600 μg/mL	2.00×10^3	3.30	27.04
800 μg/mL	3.00×10^2	2.48	20.32
1000 μg/mL	2.00×10^2	2.30	18.85
1200 μg/mL	0.00	0.00	0

4.3 Results of Virulence Test

Profuse white watery diarrhoea (Plate II and III), 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 broilers challenged with the 5-BU attenuated stock, however white diarrhoea was absent, but a normal brown pasty dropping (Plate IV and V). Post mortem observable change three days after challenge was a slightly enlarged and congested liver (Plate VI) in broilers challenged with the non-attenuated stock. Also, the mean number of *Salmonella* Kentucky re-isolated from liver of broilers challenged with the non-attenuated stock was 4.0×10^5 CFU/mL (equals 5.60 to Log_{10}) and percentage re-isolation was 62.22 % (Table 4.2). In broilers challenged with 5-BU attenuated stock, post mortem examination revealed seemingly normal liver (Plate VII) and the mean number of *Salmonella* Kentucky re-isolated in liver of broilers challenged with 5-BU attenuated isolate was 5.0×10^2 CFU/mL (equals 2.70 to Log_{10}), percentage re-isolation was 30.0 % (Table 4.2).

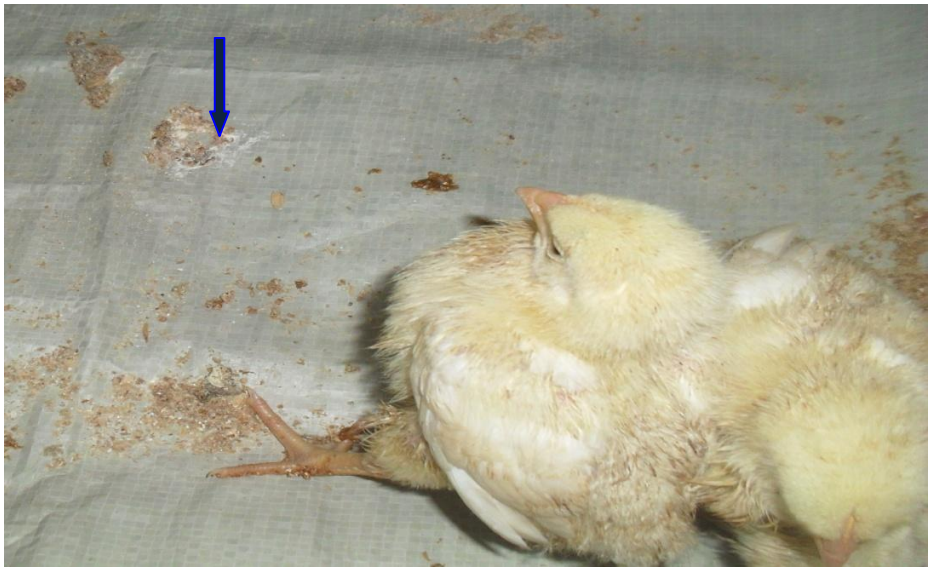


Plate II: 14-day-old chicks showing somnolence and profuse white watery diarrhoea during virulence test with field isolate administered orally.

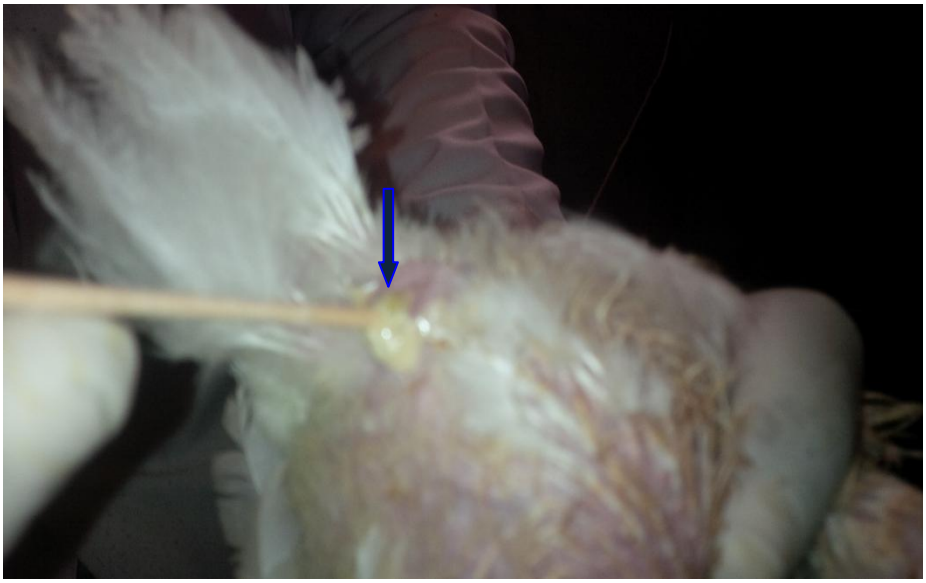


Plate III: Broiler showing profuse white watery diarrhea after oral challenge by *S.* Kentucky field isolate



Plate IV: 14-day-old chicks showing somnolence and normal formed faeces following oral challenge using 5-bromouracil attenuated *S.* Kentucky.



Plate V: Broiler showing normal formed faeces after challenge by 5-BU attenuated *S.* Kentucky



Plate VI: liver of chicks challenged with field isolate of *S.* Kentucky with mild congestion and swelling.

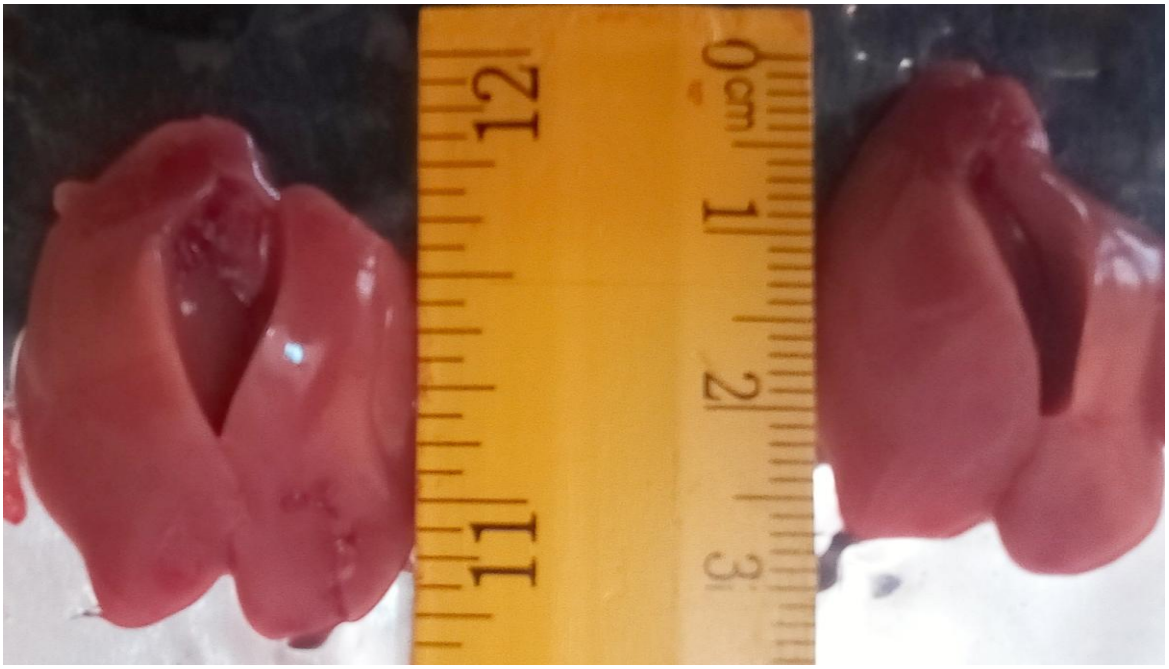


Plate VII: Normal conformations of liver of chicks challenged with 5-bromouracil attenuated isolate of *S.* Kentucky.

Table 4.2: Percentage hepatic re-isolation of *Salmonella* Kentucky following challenge with 1×10^9 CFU/mL stock cultures.

5-bromouracil concentration	**Mean CFU/mL (N)**	**$Log_{10}N \pm$ S. E. M**	**% Re-isolation ($Log_{10}N/9.0 \times 100$)**
5-BU mutated	5.0×10^2	2.70 ± 0.028	30.00
Un-mutated	4.0×10^5	5.60 ± 0.050	62.22

$P < 0.0001$

Table 4.3: Purity test undertaken on prepared live attenuated and inactivated experimental vaccines

Vaccine Type	**contaminating aerobe**	**Contaminating anaerobe**	**Contaminating Fungi**	**Sterility check**
Live	Negative	Negative	Negative	Not applicable
Killed	Negative	Negative	Negative	Negative

Table 4.4: Result of percentage (%) vaccinal efficacy against *Salmonella* Kentucky in broiler chickens after challenge

	Age of birds in days			
Group	**Day 35**	**Day 42**	**Day 49**	**Day 54**
Live attenuated	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)

No statistical significant difference ($P > 0.05$) between the Vaccinated groups.

Table 4.5: Result of the mean number of *Salmonella* Kentucky count in CFU/mL (N) re-isolated in faeces after challenge

Group	Age of birds			
	Day 35 CFU/mL	**Day 42 CFU/mL**	**Day 49 CFU/mL**	**Day 54 CFU/mL**
Live vaccine	8.80×10^3	1.28×10^3	3.50×10^2	0
Killed vaccine	9.00×10^3	2.43×10^3	4.75×10^2	3.50×10^2
Control (unvaccinated)	4.33×10^5	2.24×10^6	1.34×10^7	1.33×10^5

$P < 0.001$ when vaccinated groups were compared to control

Table 4.6: Relative percent (%) faecal inhibition of *Salmonella* Kentucky (CFU/mL) between vaccinated groups to unvaccinated group.

	Age of birds			
Vaccine group	**Day 35**	**Day 42**	**Day 49**	**Day 54**
Live attenuated vaccine	31.77	51.47	64.61	100.00
Inactivated vaccine	29.97	46.58	62.67	49.60

$P < 0.001$ when vaccinated groups were compared at day 54

4.4 Result of faecal Salmonellae shed in Broiler Chickens on sampling days Post Challenge

The mean faecal Salmonellae shed in broiler chickens between the live attenuated and inactivated vaccine group did not differ significantly ($P > 0.05$) when compared on day 35, day 42 and day 49 of age, however on day 54 of age, there was statistically significant difference seen ($P < 0.001$) between the two vaccine groups (Figure 4.1). When the vaccinated (live attenuated and inactivated) groups were compared to the unvaccinated group, there was significant difference ($P < 0.001$) seen on all sampling days; day 35, day 42, day 49 and day 54 of age post challenge (Figure 4.1).

4.5 Result of correlation for developed Antibody (IgY) titre to faecal Salmonellae shed in faeces of Vaccinated Broiler Chickens

The correlation coefficient R^2 for the mean serum antibody (IgY) titre to faecal salmonellae shed in broiler chickens immunized with the live attenuated vaccine was 0.8519. This represents a strong positive correlation (Figure 4.3). At day 35 and day 42 of age, antibody titre rose while faecal Salmonellae shed declined. At day 49 and day 54 of age there was commensurate decline in serum antibody (IgY) titre and faecal salmonellae shed (Figure 4.3). The correlation coefficient R^2 for the mean serum antibody (IgY) titre to faecal salmonellae shed in broiler chickens immunized with the inactivated vaccine was 0.3107. This represents a weak positive correlation (Figure 4.4). All sampling days post challenge; day 35, day 42, day 49 and day 54 of age were not commensurate. At day 35 and day 42 of age, the mean serum antibody (IgY) titre rose while faecal Salmonellae shed declined but on day 49 and day 54 of age, serum antibody (IgY) titre and faecal Salmonellae shed declined (Figure 4.4).

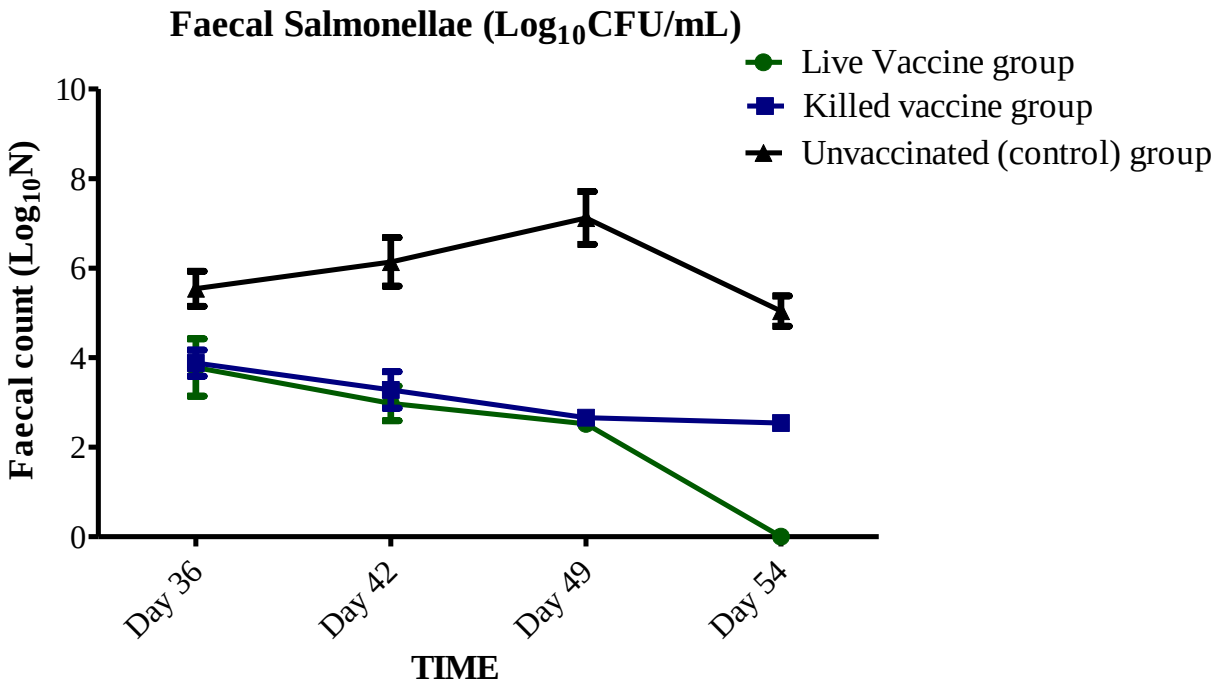


Figure 4.1: Graph for mean faecal *S.* Kentucky shed in CFU/mL to the base of Log_{10} for sampling days post challenge with field isolate in broilers chickens

Table 4.7: Result of ELISA showing the mean number in ng/mL of developed antibody (IgY) titre in serum of experimental birds

	Age of birds							
Group	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 42**	**Day 49**	**Day 54**
Live vaccine	2.71	4.44	10.51*	19.85***a**	22.15*	23.10*	22.02*	20.56*
Killed vaccine	2.44	4.46	11.47*	18.23*	20.81*	22.15*	20.70*	19.12*
Unvaccinated	2.14	3.85	3.06	3.61	8.39	11.51	8.57	8.64

* represents values P< 0.001 when vaccinated groups values were compared to the unvaccinated group.
a represents values P < 0.05 when live vaccine group were compared to killed vaccine group

Serum Dilution (1:100)

Paired sample t-Test results between live vaccine group and killed vaccine group
P value = 0.05
Correlation coefficient = 0.997

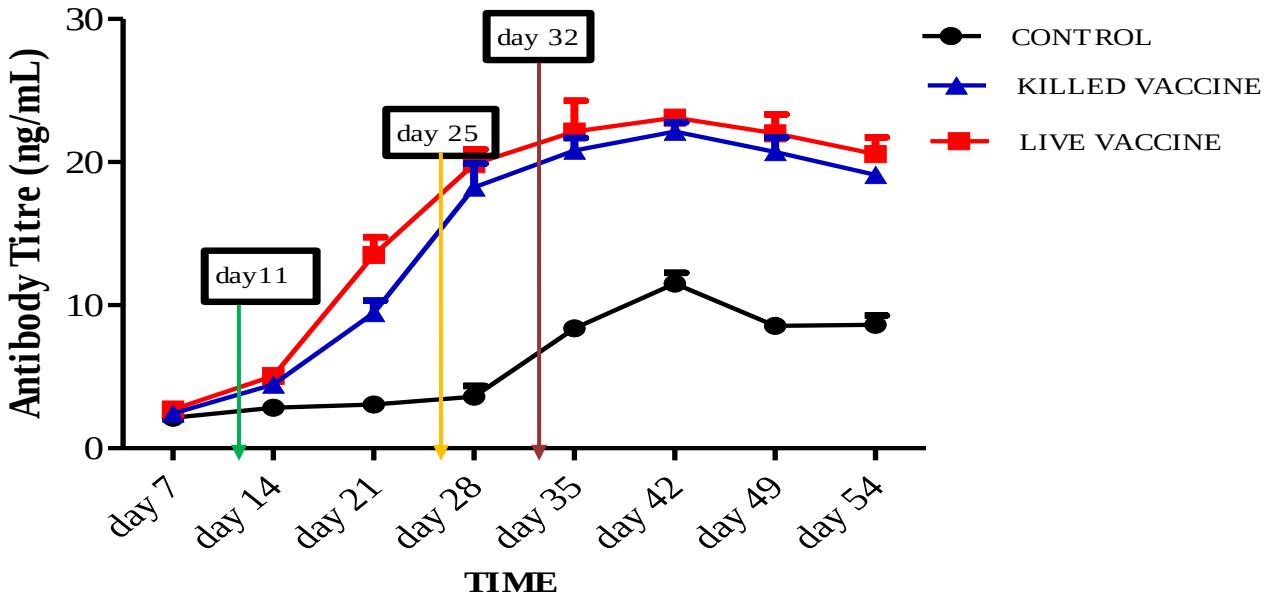


Figure 4.2: Trend for the mean immunoglobulin (IgY) levels in the sera of broiler chickens before and after immunization with experimental vaccines

Key

1st dose
2nd dose
Challenge

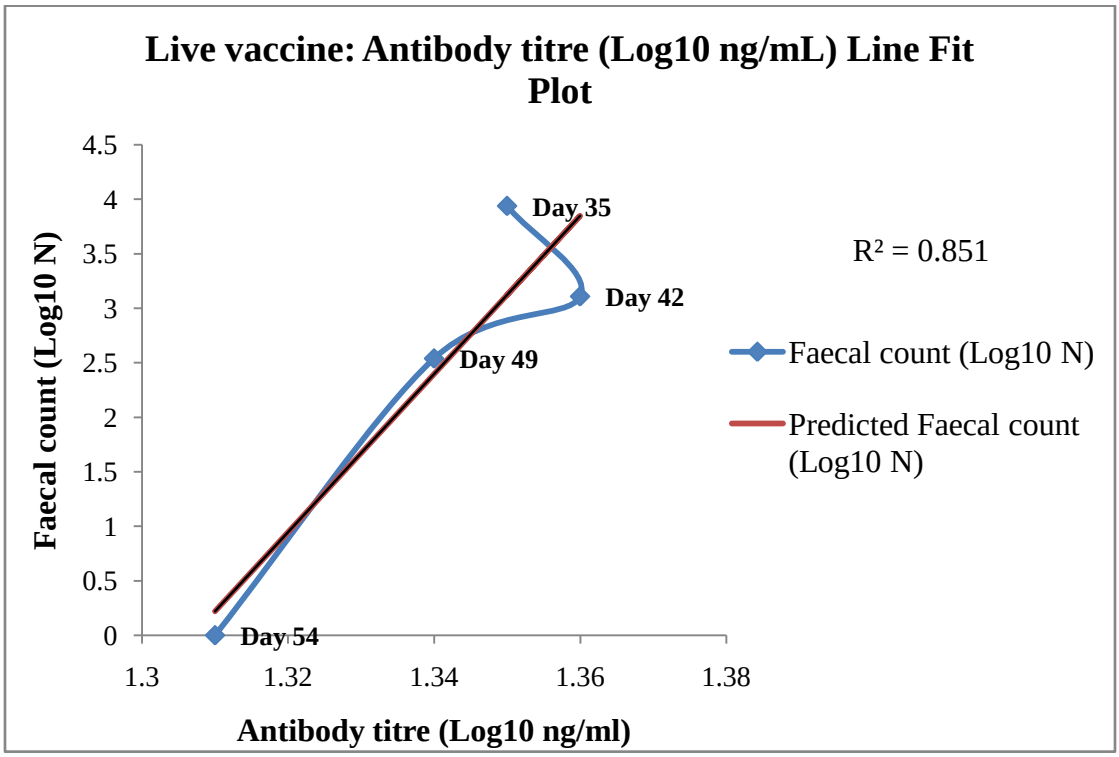


Figure 4.3: Correlation of the effect of mean Antibody (IgY) titre on the mean faecal Salmonellae shed in broiler chickens immunized with live vaccine

R^2 (0.8519): The correlation coefficient represents a strong positive relationship

P value = 0.333

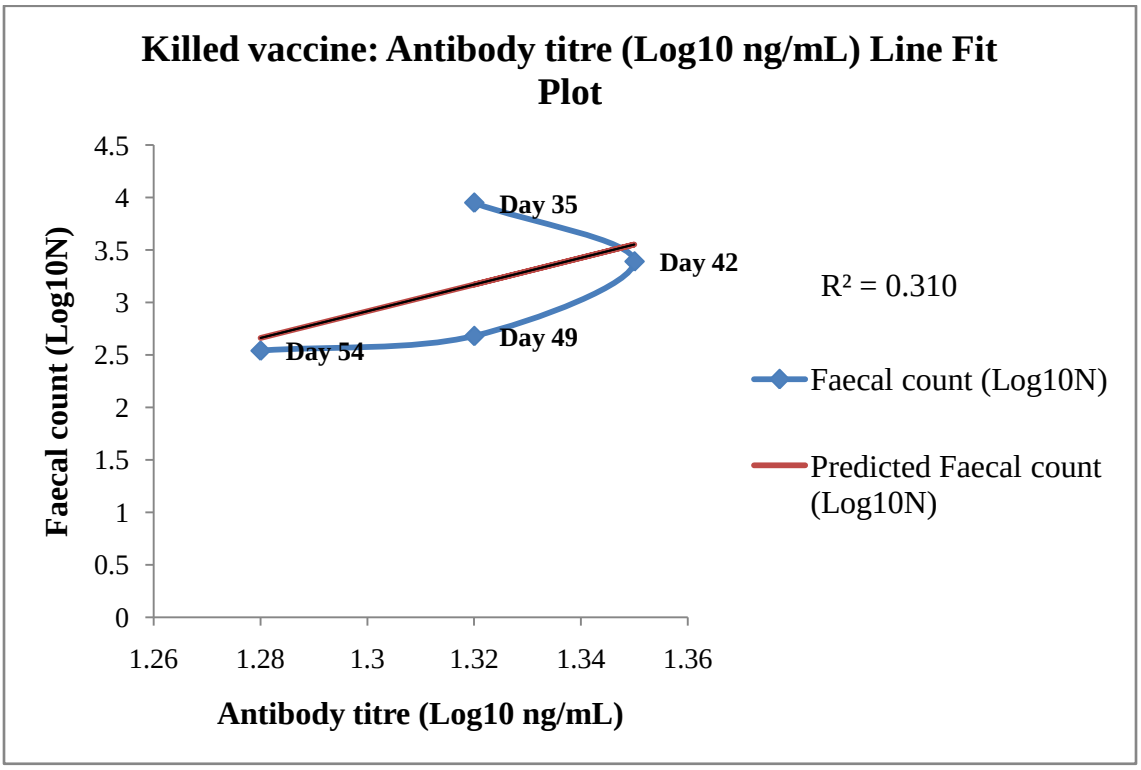


Figure 4.4: Correlation of the effect of mean Antibody (IgY) titre on the mean faecal Salmonellae shed in broiler chickens immunized with inactivated vaccine

R^2 (0.3107): The correlation coefficient represents weak positive relationship
P value = 0.417

CHAPTER FIVE

DISCUSSION

In the present study the concentration of 5-bromouracil at 1000 μg/mL was used for attenuation for the preparation of live experimental vaccine. The preparation of the experimental vaccine was carried out without investigating the alterations done by the use of this chemical on *S.* Kentucky genome. 5-bromouracil has been used to cause DNA mismatch (Griffiths *et al*., 2000), mimicking thymine or cytosine and inducing mutations during replication (Griffiths *et al*., 2000). In this study the score for attenuation was judged in respect to observable difference in clinical signs, gross hepatological changes and re-isolation of *Salmonella* from the liver. Gross hepatological lesions were congestion and hepatomegaly in birds challenged with stock culture of un-mutated isolate, these were absent in the liver of birds challenged with stock culture from 5-BU attenuated isolate, these findings were similar to Hossain *et al*. (2006). Somnolence, cuddling and anorexia were observed in the first 48 hours post challenge in both groups. The hepatic *Salmonella* Kentucky count in CFU/mL and percentage re-isolation in birds challenged with 5-BU attenuated isolate was 5×10^2 CFU/mL and 30.00 % and 4×10^5 CFU/mL and 62.22 % in birds challenged with un-mutated field isolate, these values were significantly different when compared using Chi-square test. *Salmonella* spp. carry genes involved in adhesion and invasion (Murugka *et al*., 2003) and attenuation of these genes can reduce the organism capacity to colonize and invade organs, though this was not confirmed in the present study. The noticeable differences in pathogenicity between the two challenged groups were attributed to the attenuataive effect of 5-BU on stock culture of *Salmonella* Kentucky. *Salmonella* Kentucky in this study failed to cause mortality in broilers chickens challenged either with the field isolate or experimentally 5-BU attenuated isolate. This finding contradicts the hypothesis proposed by

Mshelbwala *et al*. (2017) who reported that motile serotypes of Salmonellae may cause mortality in poultry and Nourhan *et al*. (2015) who reported 80 % mortality in chickens challenged with field isolate of *S*. Kentucky.

Broiler chickens (Zartech) used in this study showed no Salmonellae organisms in faeces from day-old to day 32 of age prior to challenge by live field isolate. Yellow colonies present (Appendix II) could be *Citrobacter* spp., *Escherichia coli* or *Proteus* spp. These micro floras are capable of fermenting xylose but incapable of fermenting lactose or sucrose and cause an acid pH in the medium therefore forming yellow colonies. Also, they lack the capacity to metabolize thiosulfate to produce hydrogen sulfide, which forms colonies with a black center as typified by *Salmonella* Kentucky (Nye *et al*., 2002; Oxoid, 2018). The presence of these genera of enterobacteriaceae does not inhibit colonization by the genus *Salmonella* (Appendix III) and therefore does not interfere with the findings and results of this study (Berchieri and Barrow, 1990; Berchieri and Barrow 1991).

The live attenuated and inactivated experimental vaccines prepared in this study (Appendix I) reduced faecal *Salmonella* shed in broiler chickens when compared to the unvaccinated group. This findings were similar to those observed by Liu *et al*. (2001), Clifton-Hadley *et al*. (2002), Papezova *et al*. (2008), Nourhan *et al*. (2015) and Groves *et al*. (2016), they reported that vaccine preparations containing either live attenuated or inactivated *Salmonella* spp. significantly reduced faecal shed of the bacteria. Also, several studies (Clifton-Hadley *et al*., 2002; Papezova *et al*., 2008; Berghaus *et al*., 2011; Revolledo *et al*., 2012) observed that preparations containing live organisms showed a superior protection in preventing colonization and environmental contamination by *Salmonella* spp. evident by a superior decline in faecal *Salmonella* shed. In this study, faecal shedding of Salmonellae organisms between the vaccinated groups from day 35 to

day 42 of age, post challenge did not differ significantly when compared, but there was statistically significant difference seen on day 54 of age, agreeing to the earlier observations made in published literature that live preparation do have a superior protection. This superior protection has been attributed to the capacity of live attenuated *Salmonella* vaccines, stimulating the secretion of mediators (cytokines, interleukin and interferon) inducing a T helper 1 response and cell-mediated immunity; this type of immune response is not induced by 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 (Lax *et al*., 1995; Cerquetti and Gherardi, 2000; Mastroeni *et al*., 2001; Husseiny and Hensel, 2005). Vaccination was also shown to account for 26.65 % of total variance in the mean faecal *Salmonella* shed when all the groups were compared and considered significant. This study therefore demonstrated that the vaccinations of broiler chickens using 5-BU live attenuated vaccine and 0.6 % formalin inactivated vaccine prior to experimental induced infection caused by *S.* Kentucky were efficacious in reducing faecal shedding in broiler chickens.

At day 7 of age, the mean antibody IgY titre in serum of experimental birds were 271 ng/mL, 244 ng/mL and 214 ng/mL for live vaccine group, inactivated vaccine group and unvaccinated group respectively. There were no difference seen between vaccinated groups and unvaccinated group, similar findings was recorded by Ferdous, (2008) and Yeasmin, (2010). The values for the mean antibody IgY titre in serum at day 21 following first immunization were 1051 ng/mL in live vaccine group and 1147 ng/mL in the inactivated vaccine group, these values were significantly different when compared to the unvaccinated group with 306 ng/mL and this higher seroconversion was sustained throughout the remainder of experiment when the vaccinated and unvaccinated groups were compared. These findings were similar to those reported by Babu *et*

al. (2003) and Tizard, (2004). The superior seroconversion in immunized chickens can be attributed to the effect of the prepared vaccines in eliciting a higher serum antibody (IgY) levels which were able to provide protection against challenge by the field isolate of *S.* Kentucky. At day 42 of age antibody (IgY) titre in serum peaked in all the groups, reaching 2310 ng/mL in birds 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 at day 35 of age, these rising values may all be related to an increase mobilization of antibody (IgY) due to the introduction of live bacteria, similar findings were observed by Nourhan *et al*. (2015).

A strong positive correlation of 0.997 was observed between the vaccinated groups 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 titre were closely matched throughout the sampling days. This was in contrast to reports by Penha Filho and Berchieri, (2012) who reported a higher level of immunoglobulin levels in birds immunized with an inactivated vaccine than a live vaccine preparation, Papezova *et al*. (2008) also reported a higher immunoglobulin titre in birds vaccinated with inactivated preparation than a live preparation. The result from this study however was similar to Babu *et al*. (2003) 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 the humoral immune response elicited in birds (Barrow, 2007), as the level of protection depends on the challenge strain/ components of the vaccine, the route of administration, the infection dose, the age of birds and the species/line of birds. Consequently it has been difficult to compare strictly the efficacy of

the vaccine preparations currently available (Barrow, 2007; Gamazo and Irache, 2007). Papezova *et al*. (2008) prepared an inactivated vaccine enriched by SPI-1 or SPI-2 proteins, these are proteins central to *Salmonella* virulence that may be responsible for the marked difference, this present study made use of saponin as an adjuvant, saponins have shown great characteristic as an immuno-adjuvant as detailed by Barbosa, (2014), other studies make use of aluminium gels and salts as adjuvant. Adjuvants are defined as a group of structurally heterogeneous compounds that enhance or modulate the immunogenicity of the associated antigens. Despite the recognition of many different types of adjuvant, 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 (Gamazo and Irache, 2007). Little and isolated information explains how vaccines work and the immune response depending on the antigen and route of vaccination (Revolledo, 2012).

The Correlation of the effect of the mean antibody (IgY) titre on the mean faecal Salmonellae shed from day 35 to day 54 of age post challenge showed that, in the group immunized with live vaccine, R^2 equaled 0.8519. This represents a strong positive correlation and that 85.19 % of the variance in the mean faecal *Salmonella* shed can be explained by variations in the mean antibody (IgY) titre. This was superior to that observed in the inactivated vaccine group with an R^2 of 0.3107 representing a weaker degree of positive correlation and meaning that 31.07 % of the variance in the mean faecal *Salmonella* shed can be explained by variations in the mean antibody (IgY) titre, also all the four points representing the sampling days post challenge were not commensurate in the inactivated vaccine group. Overall, immunized birds maintained a 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 groups were compared to the control. This finding agrees with (Clifton-Hadley *et al*., 2002; Khan *et al*., 2003; Papezova *et al*., 2008; Berghaus *et al*., 2011; Nourhan *et al*., 2015) who established in experimental studies on birds that vaccinated birds had significantly higher *Salmonella* specific antibody titres than did unvaccinated birds and significant lower prevalence and load. Also, Tizard, (2004) stated that antibodies are effective opsonins that can mediate phagocytosis through antibody receptors which are specific for phagocytic cells. Therefore it can be inferred that the antibody response contributes to the clearance and protection against colonization by *S.* Kentucky in the gastrointestinal tracts of vaccinated birds.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Based on the results from this study, the following conclusions were drawn;

1. 5-bromouracil at concentration of 1000 µg/mL adequately attenuated stock culture of *Salmonella* Kentucky without apparent adverse effect or mortality in broilers chickens when used in preparation of vaccine.
2. 0.6 % formalin can adequately inactivate *Salmonella* Kentucky without apparent adverse effect or mortality in broilers chickens when used in preparation of vaccine.
3. There was 100 % vaccinal efficacy and inhibition of *S.* Kentucky by the live vaccine preparation and 82.82 % vaccinal efficacy and 49.60 % inhibition by the inactivated vaccine preparations.
4. The interplay between vaccination regimens that include immunization with either 5-bromouracil attenuated live *Salmonella* Kentucky vaccine or 0.6 % formalin inactivated *Salmonella* Kentucky vaccine and developed antibody (IgY) titre can reduce caecal colonization and interfere with the pattern and load of shed of *S.* Kentucky in faeces of broiler chickens

6.2 Recommendations

The following recommendations were made from this study:

1. Further quality control studies should be carried out on developed experimental vaccines to ascertain longevity, shelf life and safety period.
2. A more elaborate and extensive studies should be carried out on birds to ascertain the Period/duration of protection of developed protective antibody elicited in immunized birds.
3. The developed vaccines can be incorporated into the current Fowl typhoid vaccine produced by the National Veterinary Research Institute, Vom, Nigeria to produce a bivalent vaccine and made ready for farm use.
4. Further investigative studies and complete evaluation of humoral immune response (IgM, IgY and IgA), and cell mediated immunity (especially $CD8^+$ cells) elicited by the Live vaccine be undertaken.

REFERENCES

Adeleke, M. A., Akatah, H. A., Hassan, A. O. and Adebimpe, W. O. (2012). Microbial load and multiple drug resistance of pathogenic bacteria isolated from feaces and body surfaces of cockroaches in an urban area of Southwestern Nigeria. *Journal of Microbiology Biotechnology and Food Science,* 1: 1448-1461.

Alphons, J. A. and Jaap-van Dijk, E. (2005). Distribution of "Classic" virulence factors among *Salmonella* spp. *FEMS Journal Immunology and Medical Microbiology,* 44: 251-259.

Ao, T. T., Feasey, N. A., Gordon, M. A., Keddy, K. H., Angulo, F. J. and Crump, J. A. (2015). Global burden of invasive nontyphoidal *Salmonella* disease. *Emerging Infectious Diseases*, 21: 941- 945.

Arpaia, N., Godec, J., Lau, L., Sivick, K. E., McLaughlin, L. M., Jones, M. B., Dracheva, T. Peterson, S. N., Monack, D. N. and Barton, G. M. (2011). TLR signaling is required for *Salmonella* Typhimurium virulence. *Cell*, 144: 675 - 688.

Atterbury, R. J., Davies, R. H., Carrique-mas, J. J., Morris, V., Harrison, D., Tucker, V. and Allen, V. M. (2010). Effect of delivery method on the efficacy of *Salmonella* vaccination in chickens. *The Veterinary Record,* 167: 161-164.

Atterbury, R. J., Carrique-Mas, J. J., Davies, R. H. and Allen, V. M. (2009). *Salmonella* colonisation of laying hens following vaccination with killed and live attenuated commercial *Salmonella* vaccines. *Veterinary Records,* 165: 493–496.

Aziz, M. A., Midha, S., Waheed, S. M. and Bhatnagar, R. (2007). Oral vaccines: new needs, new possibilities. *BioEssays*, 29: 591-604.

Babu, U., Dalloul, R. A., Okamura, M., Lillehoj, H. S., Xie, H., Raybourne, R. B., Gaines, D. and Heckert, R. A. (2004) "*Salmonella* Enteritidis clearance and immune response in Chickens following *Salmonella* vaccination and challenge. *Veterinary Immunology and Immunopathology*, 101: 251-257.

Babu, U., Scott, M. and Myers, M. J. (2003). Effects of live attenuated and killed *Salmonella* vaccine on T-lymphocyte mediated immunity in laying hens. *Veterinary Immunology and Immunopathology*, 91:39–44.

Barbosa, A. P. (2014). Saponins as immunoadjuvant agent: A Review. *African Journal of Pharmacy and Pharmacology*, 8(41): 1049 – 1057.

Barrow P. A. (2007). *Salmonella* infections: immune and non-immune protection with vaccines. *Avian Pathology*, 36: 1-13.

Barrow, P. A. and Wallis, T. S. (2000) Vaccination against *Salmonella* infections in food animals: rationale theoretical basis and practical applications In: editors Wray C, Wray A., *Salmonella* in domestic animals. New York: CABI Publishing. pp. 323-339

Barua, H., Biswas, P. K., Olsen, K. E. P., Shil, S. K. and Christensen, J. P. (2013). Molecular characterization of motile serovars of *Salmonella enterica* from breeder and commercial broiler poultry farms in Bangladesh. *PLoS ONE,* 8(3): 1 – 9.

Bavishi, C. and Dupont, H. L. (2011). Systematic review: The use of proton pump inhibitors and increased susceptibility to enteric infection. *Alimentary Pharmacology,* 34: 1269 – 1281.

Baxter, V. A. (2015). Assessment of killed *Salmonella* vaccine efficacy in broiler breeders and their progeny: *MSc thesis*; University of Georgia, Athens, Georgia. Pp. 1 – 46.

Belyakov, I. M. and Ahlers, J. D. (2009). What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? *The Journal of Immunology,* 183: 6883 – 6892.

Berchieri, A. and Barrow, P. A. (1990). Further studies on the inhibition of colonization of the chicken alimentary tract with *Salmonella* Typhimurium by pre-colonization with an avirulent mutant strain. *Epidemiology and Infection*, 104: 427– 441.

Berchieri, A. and Barrow, P. A. (1991). In vitro characterization of intra-generic inhibition of growth in *Salmonella* Typhimurium. *Journal of General Microbiology,* 137: 2147–2153.

Berghaus, R. D., Thayer, S. G., Maurer, J. J. and Hofacre, C. L. (2011). Effect of vaccinating breeder chickens with a killed *Salmonella* vaccine on *Salmonella* prevalence and loads in breeder and broiler chicken flocks. *Journal of Food Protection*, 74: 727-734.

Berndt, A. and Methner, U. (2001). Gamma/delta T cell response of chickens after oral administration of attenuated and non-attenuated *Salmonella* Typhimurium strains. *Veterinary Immunology and Immunopathology,* 78: 143–161.

Berndt, A., Pieper, J. and Methner, U. (2006). Circulating γδ T cells in response to *Salmonella enterica* serovar Enteritidis exposure in chickens. *Infection and Immunity*, 74: 3967–3978.

Berthelot-hérault, F., Mompart, F., Zygmunt, M. S., Dubray, G. and Duchet-suchaux, M. (2003). Antibody responses in the serum and gut of chickens lines differing in cecal carriage of *Salmonella* Enteritidis. *Veterinary Immunology and Immunopathology,* 96: 43- 52.

Bettridge, J. M., Lunch, S. E., Brena, M. C., Melese, K., Dessie, T., Terfa, Z. G., Desta, T. T., Rushton, S., Hanotte, O., Kaiser, P., Wigley, P. and Christley, R. M. (2014). Infection-interactions in Ethiopian village chickens. *Preventive Veterinary Medicine*, 117: 358-366.

Beutlich, J., Guerra, B., Schroeter, A., Arvand, M., Szabo, I. and Helmuth, R. (2012). Highly ciprofloxacin resistant *Salmonella enterica* serovar Kentucky isolates in turkey meat and a human patient. *Berlin and Munchen Tierarzotil Wochenscher.* 125: 89 – 95.

Bhan, M. K., Bahl, R., Sazawal, S., Sinha, A., Kumar, R., Mahalanabis, D. and Clemens, J. D. (2002). Association between *Helicobacter pylori* infection and increased risk of typhoid fever. *Journal of Infectious Disease*, 186: 1857 – 1860.

Blanc-Potard, A. B. and Groisman, E. A. (1997). The *Salmonella* selC locus contains a pathogenicity island mediating intramacrophage survival. *EMBO Journal*, 16; 5376 - 5385.

Bohez, L., Ducatelle, R., Pasmans, F., Botteldoorn, N., Haesebrouck, F. and Van Immerseel, F. (2006) *Salmonella enterica* serovar Enteritidis colonization of the chicken caecum requires the *hilA* regulatory protein. *Veterinary Microbiology*, 116: 202-210.

Borsoi, A., Rushel do Santos, L., Rodrigues, L. B., Hamilton, L. S., Salle, C. T. P. and Vldamir, P. N. (2011). Behaviour of *Salmonella* Heidelberg and *Salmonella* Enteritidis strains following Broiler chick inoculation: Evaluation of cecal morphometry, Liver and cecum Bacteria counts and fecal excretion patterns. *Brazilian Journal of Microbiology*, 42(1): 266 – 273.

Bouvet, J. P., Decroix, N. and Pamosinlapatham, P. (2002). Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends in Immunology*, 23: 209-213.

Boyd, D., Peters, G. A., Cloeckaert, A., Boumedine, K. S., Chaslus-Dancla, E and Imberechts, H. (2001). Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *Journal of Bacteriology*, 183: 5725–5732.

Brenner, F. W. (1998). Modified Kauffmann-White scheme. Centers for Disease Control and Prevention, Atlanta, GA.

Bronzan, R. N., Taylor, T. E., Mwenechanya, J., Tembo, M., Kayira, K., Bwa-naisa, L., Njobvu, A., Kondowe, W., Chalira, C., Walsh, A. L., Phiri, A., Wilson, L. K., Molyneux, M. E. and Graham, S. M. (2007). Bacteremia in Malawian Children with severe malaria: prevalence, etiology, HIV co-infection and outcome. *Journal of Infectious Disease.* 195: 895 – 904.

Callaway, T. R., Edrington, T. S., Anderson, R. C., Byrd, J. A., and. Nisbet, D. J. (2008). Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella*. *Journal of Animal Science*, 86: E163–E172.

Center for Disease Control and Prevention (CDC) (2008). *Salmonella* surveillance: Annual summary, 2006. U.S. Department of Health and Human Services, CDC, Atlanta, GA.

Center for Disease Control and Prevention (CDC) (2011). National Center for Emerging and Zoonotic Infectious Diseases. Enteric Diseases Epidemiology Branch; *Salmonella* surveillance overview. pg. 1-12.

Center for Food Security and Public Health (CFSPH) (2009). Fowl typhoid and pullorum disease; bacillary white diarrhea (Pullorum disease). CFSPH, College of Veterinary Medicine, Iowa state university. Pp. 1 -5.

Cerquetti, M. C. and Gherardi, M. M. (2000). Vaccination of chickens with a temperature sensitive mutant of *Salmonella* Enteritidis. *Vaccine,* 18: 1140 -1145.

Chabalgoity, J. A., Baz, A., Rial, A. and Grille, S. (2007). The relevance of cytokines for development of protective immunity and rational design of vaccines. *Cytokine and Growth Factor Review* 18: 195-207.

Chaussé, A. M., Grepinet, O., Bottreau, E., Le vern, Y., Menanteau, P., Troterau, J., Robert, V., Wu, Z., Kerboeuf, D., Beaumont, C. and Velge, P. (2011). Expression of TLR4 and downstream effetors in selected cecal cell subpopulations of chicks resistant or susceptible to *Salmonella* carrier-state. *Infection and Immunity*, doi: 10.1128/IAI.00025-11.

Chia, T. W. R., Goulter, R. M., McMeekin, T., Dykes, G. A. and Fegan, N. (2009). Attachmnet of different *Salmonella* serovars to materials commonly used in a poultry processing plant. *Food Microbiology*, 26: 853 – 859.

Clifton-Hadley, F., Breslin, M. and Venable, L. M. (2002). A laboratory study of an inactivated bivalent iron restricted *Salmonella enterica* serovars Enteritidis and Typhimurium dual vaccine against Typhimurium challenge in chickens. *Veterinary Microbiology,* 89: 167–179.

Clinical Laboratory Standards Institute (CLSI). (2013). Antimicrobial susceptibility of *Salmonella;* performance standards for antimicrobial susceptibility testing: twenty-third informational supplement. Wayne, PA, USA. Document M100 – S23.

Collinson, K., Doig, P. C., Doran, J. L., Clouthier, S., Trust, T. J. and Kay, W. W. (1993). Thin aggregative fimbriae mediate binding of *Salmonella* Enteritidis to fibronectin. *Journal of Bacteriology,* 175(1): 12-18.

Coombes, J. L. and Powrie, F. (2008). Dendritic cells in intestinal immune regulation. *Nature Reviews Immunology*, 8: 435 - 446.

Crhanova, M., Hradecka, H., Faldynova, M., Matulova, M., Havlickova, H., Sisak, F. and Rychlik, I. (2011). Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* serovar *Enteritidis* infection. *Infection and Immunity*, doi:10.1128/IAI.01375-10.

Crump, J. A., Sjolund-Karlsson, M., Gordon, M. A. and Parrye, M. C. (2015). Epidemiology, Clinical presentation, laboratory diagnosis, antimicrobial resistance and antimicrobial management of invasive *Salmonella* infections. *Clinical Microbiology Review*, 28(4): 901 – 936.

Curtiss, R., Porter, S. B. and Munson, M. (1991). Nonrecombinant and recombinant avirulent *Salmonella* live vaccines for poultry. In: Blankenship L. C., Bailey, J. S., Cox, N. A., Stern, N. J., Meinersmann, R. J. editors. Colonization Control of Human Bacterial Enteropathogens in Poultry. New York, New York: Academic Press. pp. 169–198.

Deen, J., von Seidlein, L., Andersen, F., Elle, N., White, N. J. and Lubell, Y. (2012). Community acquired bacterial bloodstream infections in developing countries in South and Southeast Asia: A systematic review. *Lancet Infectious Disease*, 12: 480 – 487.

Den-Baker, H. C., Switt, A. I. and Govoni, G. (2011). Genome sequence reveals diversification of virulence factor content and possible host adaptation in distinct sub populations of *Salmonella enterica*. *BMC Genomics*. 12: 425 – 432.

Desmidt, M., Ducatelle, R., Mast, J., Goddeeris, B. M., Kaspers, B. and Haesebrouck, F. (1998). Role of the humoral immune system in *Salmonella* enteritidis phage type four infection in chickens. *Veterinary Immunology and Immunopathology*, 63: 355 - 367.

Donlan, R. M. and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15(2): 167-193.

Doublet, B., Praud, K., Bertrand, S., Collard, J. M., Weill, F.X and Cloeckaert, A. (2008). Novel insertion sequence- and transposon-mediated genetic rearrangements in genomic island SGI1 of *Salmonella enterica* serovar Kentucky. *Antimicrobial Agents Chemotherapy*, 52: 3745–3754.

Efstathios, G., Nikos, C., Panagiotis, S. and George, J. N. (2012). Attachment and Biofilm Formation by *Salmonella* in food processing Environments; *Salmonella a Dangerous Foodborne Pathogen*, 8:157 – 180.

Fagbamila, I. O., Barco, L., Mancin, M., Kwaga, J., Ngulukun, S. S., Zavagnin, P., Lettini, A. A., Lorenzetto, M., Abdu, P. A., Kabir, J., Umoh, J., Ricci, A. and Muhammad, M. (2017). *Salmonella* serovars and their distribution in Nigerian commercial chicken layer farms. *PLoS ONE Journal*, 12(3): 1 – 15.

Farmer, J. J. III (1999). Enterobacteriaceae. In Murray, P. R., Baron, E. J., Pfaller, M. A. Tenover, F. C. and Yolken, R. H. (ed). *Manual of Clinical Microbiology*, 7th edition. ASM Press, Washington, D. C. pp. 442 – 458.

Fasure, A. K., Deji-Agboola, A. M. and Akinyemi, K. O. (2012). Antimicrobial resistance patterns and emerging fluoroquinolone resistant *Salmonella* isolates from poultry and asymptomatic poultry workers. *African Journal of Microbiology Research*, 6: 2610-2615.

Feasey, N. A., Archer, B. N., Heyderman, R. S., Sooka, A., Dennis, B. and Gordon, M. A. (2010). Typhoid fever and invasive nontyphoidal salmonellosis, Malawi and South Africa. *Emerging infectious Disease*, 16: 1448 – 1451.

Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S. and Gordon, M. A. (2012) Invasive non typhoidal *Salmonella* disease: An emerging and neglected tropical disease in Africa. *The Lancet*, 379: 2489-2499.

Ferdous, J. (2008). Immunogenicity study of DLS prepared *Salmonella* Gallinarum vaccine in comparison to commercially available one in layer chicken. *M. S. Thesis* submitted to the Department of Microbiology and Hygiene, Faculty of Veterinary Sciences, Bangladesh Agricultural University, Mymensingh, pp. 38 – 39.

Foley, L. Steven., Rajesh Nayak., Irene, B. Hanning., Timothy J. Johnson, Jing Han, and Steven C. Ricke (2011). Population Dynamics of *Salmonella enterica* Serotypes in Commercial Egg and Poultry Production. *Applied and Environmental Microbiology,* pp. 4273-4279.

Foley, S. L., Lynne, A. M. and Nayak, R. (2008). Salmonella challenges: Prevalence in swine and poultry and potential Pathogenicity of such isolates. *Journal of Animal Science*, 86: E149–E162.

Food and Agricultural Organization (FAO), (2006). The structure and importance of the commercial and village based poultry industry in Nigeria. Adene D. F and Oguntade, A. E.

Food and Drug Administration. (FDA) (2010). National Antimicrobial Resistance Monitoring System enteric bacteria (NARMS): 2007 executive report. U.S. Department of Health and Human Services, FDA, Rockville, MD.

Food and Drug Administration. (FDA) (2008). "Food Safety A – Z reference guide – *Salmonella*"

Fox, A. (2011). *Microbiology and Immunology Online*. *Enterobacteriacea,*Vibrio, Campylobacter and Helicobacter. Retrieved on May 13, 2012, from Pathmicro. Med.sc.edu/fox/enterobact.htm.

Fricke, W. F., McDermott, P. F., Mammel, M. K., Zhao, S. and Johnson, T. J. (2009) Antimicrobial resistance-conferring plasmids with similarity to virulence plasmids from avian pathogenic *Escherichia coli* strains in *Salmonella enterica* serovar Kentucky isolates from poultry. *Applied Environmental Microbiology,* 75: 5963–5971.

Fuche, F. J., Sow, O., Simon, R. and Tennant, S. M. (2016). *Salmonella* Serogroup C: Current Status of Vaccines and Why They Are Needed. *Clinical Vaccine and Immunology,* 23(9): 737-45

Gala'n, J. E. and Wolf-Watz, H. (2006). Protein delivery into eukaryotic cells by type III secretion machines. *Nature,* 444:567–573.

Gal-mor, O., Boyle, E. C. and Grassl, G. A. (2014). Same species, different diseases: how and why typhoidal and non typhoidal *Salmonella enterica* serovars differ. *Front Microbiology,* 5: 391 - 399.

Gamazo, C. and Irache, J. M. (2007). *Salmonella* Vaccines. In: Mendez-Vilas, A. (Ed) *Commmunicationg Current Research and Educational Topics and Trends in Applied Microbiology,* 518 – 524.

Gast, R. K. (2007). Serotype-specific and serotype independent strategies for preharvest control of foodborne *Salmonella* in poultry. *Avian Diseases,* 51: 817- 828.

Gast, R. K. and Beard, C. W. (1990). "Production of *Salmonella* Enteritidis contaminated eggs by experimentally infected hens. *Avian Diseases,* 34: 438 – 446.

Gast, R. K., Stone, H. D. and Holt, P. S. (1993). Evaluation of the efficacy of oil emulsion bacterins reducing fecal shedding of *Salmonella* Enteritidis by laying hens. *Avian Diseases,* 37: 1085 – 1091.

Giaouris, E. D. and Nychas G. J. E. (2006). The adherence of *Salmonella* Enteritidis PT4 to stainless steel: the importance of the air-liquid interface and nutrient availability. *Food Microbiology,* 23: 747-752.

Giaouris, E., Chorianopoulos, N. and Nychas, G. J. E. (2005). Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica* Enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection,* 68: 2149-2154.

Gilbert, P., Allison, D. G. and McBain, A. J. (2002). Biofilms *in vitro* and *in vivo*: Do singular mechanisms imply cross-resistance. *Journal of Applied Microbiology,* 92: 98 – 110.

Gordon, M. A. (2008). *Salmonella* infections in immune compromised adults. *Journal of Infection,* 56: 413 – 422.

Gordon, M. A., Graham, S. M., Walsh, A. L., Wilson, L., Phiri, A., Molyneux, E., Zijlstra, E. E., Heyderman, R. S., Hart, C. A. and Molyneux, M. E. (2008). Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clinical Infectious Disease,* 46: 963 – 969.

Gordon, M. A., Kankwatira, A. M. K., Mwafulirwa, G., Walsh, A. L., Hopkins, M. J., Parry, C. M., Faragher, E. B., Zijlstra, E. E., Heyderman, R. S. and Molyneux, M. E. (2010). Invasive nontyphoidal *Salmonella* establish systemic intracellular infection in HIV infected adults: an emerging disease pathogenesis. *Clinical Infectious Disease,* 50: 953 – 962.

Griffiths, A. J. F., Suzuki, D. T., Miller, J. H., Lewontin, R. C. and Gelbart, W. M. (2000). An Introduction to Genetic Analysis 7th edition. New York: W. H. Freeman and Company. NBK21936.

Groves, P. J., Sharpe, S. M., Muir, W. I., Pavic, A. and Cox, J. M. (2016). Live and inactivated vaccine regimens against caecal *Salmonella* Typhimurium colonisation in laying hens. *Australian Veterinary Journal*, 94(10): 387–393.

Guard-Petter, J. (2001). The chicken, the egg and Salmonella Enteritidis. *Enviromental Microbiology*, 3(7): 421 – 430.

Guibourdenche, M., Roggentin, P., Mikoletit, M., Fields, P. I., Bockemuhl, J., Grimont, P. A. D. and Weill, F. X. (2010). Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. *Research in Microbiology*, 161: 26-29.

Guiney, D. G., Fang, F. C., Krause, M. and Libby, S. (1994). Plasmid-mediated virulence genes in nontyphoid *Salmonella* serovars. *FEMS Microbiology Letter,* 124: 1 – 9.

Gulig, P.A., Danbara, H., Guiney, D. G., Lax, A. J., Norel, F. and Rhen, M., (1993). Molecular analysis of spv virulence genes of the *Salmonella* virulence plasmids. *Molecular Microbiology*, 7: 825 - 830.

Haley, B. J., Kim, S. W., Pettengill, J., Luo, Y., Karns, J. S. and Van Kessel, J. A. S. (2016) Genomic and Evolutionary Analysis of Two *Salmonella enterica* Serovar Kentucky Sequence Types Isolated from Bovine and Poultry Sources in North America. *PLoS ONE Journal* 11(10): e0161225. doi:10.1371.

Handzlik, J., Matys, A. and Kiec´-Kononowicz, K. (2013). Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria *S. aureus. Antibiotics*, 2: 28 - 45.

Haneda, T., Okada, N., Nakazawa, N., Kawakami, T. and Danbara, H. (2001). Complete DNA sequence of comparative analysis of the 50-kilobase virulence plasmid of *Salmonella eneterica* serovar Cholerasuis. *Infection and Immunology*, 69: 2612 – 2620.

Hansen-Wester, I. and Hensel, M. (2001). *Salmonella* Pathogenicity Islands encoding Type III secretion systems. *Microbial Infection*, 3: 549 – 559.

Hayward, R. D. and Koronakis, V. (2002). Direct modulation of the host cell cytoskeleton by *Salmonella* actin-binding proteins. *Trends Cell Biology*, 12: 15–20.

Hensel, M. (2000). *Salmonella* Pathogenicity Island 2. *Molecular Microbiology*, 36: 1015 – 1023.

Herrero-Fresno, A., Wallrodt, I., Leckitcharoenphon, P., Oslen, J. E., Aarestrup, F. M. and Hendriksen, R. S. (2014). The role of the st313-td gene in virulence of *Salmonella* Typhimurium ST313. *PLos One Journal*, 9:e84566.

Hertman, I., Markenson, J and Michael, A. (1978). Fowl cholera vaccine and its preparation. Vineland Laboratories Limited, United States of America.

Hoelzer, K., Switt, A. I. M. and Wiedmann, M. (2011). Animal contact as a source of human non typhoidal Salmonellosis. *Veterinary Research,* 42(1): 34 – 38.

Holt, P. S., Gast, R. K. and Kelly-Aehle, S. (2003). Use of a live attenuated *Salmonella* Typhimurium vaccine to protect hens against *Salmonella* Enteritidis infection while undergoing moulting. *Avian Disease,* 47: 656 – 661.

Hood, S. K. and Zottola, E. A. (1997). Growth media and surface conditioning influence the adherence of *Pseudomonas fragi, Salmonella* Typhimurium and *Listeria monocytogenes* cells to stainless steel. *Journal of Food Protection,* 60: 1034 – 1103.

Hossain, M. S., Chowdhury, E. H., Islam, M. M., Haider, M. G. and Hossain, M. M. (2006). Avian *Salmonella* Infection: Isolation and Identification of Organisms and Histopathological Study. *Bangladesh Journal of Veterinary Medicine,* 4(1): 7 – 12.

Hueck C. J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology Molecular Biology Review,* 62: 379–433.

Husseiny, M. I. and Hensel, M. (2005). Evaluation of an intracellular-activated promoter for the generation of live *Salmonella* recombinant vaccines. *Vaccine,* 23: 2580 - 2590.

International Organization for Standardization (ISO) (2002). ISO 6579: 2002/FDAmd 1. Annex D. Detection of *Salmonella* species in animal faeces and in samples of the primary production stage. International Organization for Standardization ISO, Geneva.

Issenhuth-Jeanjean, S., Roggentin, P., Mikoleit, M., Guibourdenche, M., Pinna, E, Nair, S., Fields, P.I., and Weill, F-X. (2014). Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme. *Research in Microbiology,* 165: 526-530.

Iwasaki, A. and Metzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology,* 5: 987- 995.

Jeurissen, S. H. M., Lewis, F., Van Der Klis, J. D., Mroz, Z., Rebel, J. M. J. and Huurne, A. A. (2002). Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. *Current Issues in Intestinal Microbiology,* 3:1-14.

Jiang, Y., Kulkarni, R. R., Parreira, V. R., Poppe, C., Roland, K. L. and Prescott, J. F. (2010). Assessment of 2 *Salmonella enterica* serovar Typhimurium-based vaccines against necrotic enteritis in reducing colonization of chickens by *Salmonella* serovars of different serogroups. *Canadian Journal Veterinary Research*, 274: 264 – 270.

Jingjing. S., Ziqing, D. and Aixin, Y. (2014). Bacterial multidrug efflux pumps: Mechanism, Physiology and Pharmacological exploitations. *Biochemical and Biophysical Research Communications,* 453: 254 – 267.

Johnson, T. J., Thorsness, J. L., Anderson, C. P., Lynne, A. M. and Foley, S. L. (2010) Horizontal Gene Transfer of a ColV Plasmid Has Resulted in a Dominant Avian Clonal Type of *Salmonella enterica* Serovar Kentucky. *PLoS ONE Journal*, 5(12): 1 - 10.

Jones, T. F., Ingram, L. A., Cieslak, P. R., Vugia, D. J., Tobin-D'Angelo, M., Hurd, S., Medus, C., Cronquist, A. and Angulo, F. J. (2008). Salmonellosis outcomes differ substantially by serotype. *Journal of Infectious Disease*, 198: 109 – 114.

Jones, Y. E., Mclaren, I. M. and Wray, C. (2000). Laboratory Aspects of Salmonellae. IN: Wray, C. and Wray, A. (eds), *Salmonella* in domestic animals. ABI publishing, Wallingford, UK, pp. 393 – 405.

Kariuki, S., Revathi, G., Kariuki, N., Kiiru, J., Mwituria, J., Muyodi, J., Githinji, J. W., Kagendo, D., Munyalo, A. and Hart, C. A. (2006). Invasive multi drug resistant non typhoidal *Salmonella* infections in Africa: Zoonotic or Anthroponotic transmission? *Journal of Medical Microbiology*, 55: 585 – 591.

Khan, M. I., Fadl, A. A. and Venkitanarayanan, K. S. (2003). Reducing colonization of *Salmonella* Enteritidis in chicken by targeting outer membrane proteins. *Journal of Applied Microbiology*, 95: 142–145.

Kingsley, R. A., Msefula, C. L., Thompson, N. R., Kariuki, S., Holt, K. E., Gordon, M. A., Harris, D., Clarke, L., Whitehead, S., Sangal, V., Marsh, K., Achtman, M., Molyneux, M. E., Cormican, M. and Dougan, G. (2009). Epidemic multidrug resistant *Salmonella* Typhimurium causing invasive disease in sub Saharan Africa have a distinct genotype. *Genome research*, 19: 2279 – 2287.

Klochko, A. (2016) Salmonellosis. Overview, Presentation, Diagnosis, Workup, Treatment, Medication and Follow-up. Editor: Bronze, S. M. Drugs and Disease; Infectious Diseases. *Medscape*, Updated: October 21

Kokosharov, T. (2002) Clinical and hematological effects of Salmonella gallinarum endotoxin in cockerels. *Veterinary archive*, 72: 269 - 276.

Kong, Q., Liu, Q., Jansen, A. M. and Curtiss, R. (2010). Regulated delayed expression of rfc enhances the immunogenicity and protective efficacy of a heterologous antigen delivered by live attenuated *Salmonella enterica* vaccines. *Vaccine*, 28: 6094 – 6103.

Kong, W., Wanda, S. Y. and Zhang, X. (2008). Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment. *Proc National Academia of Science U S A*, 105: 9361– 9366.

Konjufca, V., Wanda, S. Y., Jenkins, M. C. and Curtiss, R. (2006). A recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine encoding *Eimeria acervulina* antigen offers protection against *E. acervulina* challenge. *Infectious Immunology*, 74: 6785 – 6796.

Kuchma, S. L. and O'Toole, G. A. (2000). Surface-induced and biofilm-induced changes in gene expression. *Current Opinion in Biotechnology*, 11: 429-433.

Kuhle, V. and Hensel, M. (2004) Cellular microbiology of intracellular *Salmonella enterica*: functions of the Type III secretion system encoded by *Salmonella* Pathogenicity Island. *Cellular Molecular Life Science,* 61: 2812 – 2826.

Kulkarni, R. R., Parreira, V. R., Jiang, Y. F. and Prescott, J. F. (2010). A live oral recombinant *Salmonella enterica* serovar Typhimurium vaccine expressing Clostridium perfringens antigens confers protection against necrotic enteritis in broiler chickens. *Clinical Vaccine Immunology*, 17: 205–214.

Kwon, H. J. and Cho, S. H. (2011). Pathogenicity of SG 9R, a rough vaccine strain against fowl typhoid. *Vaccine*, 29: 1311-1318.

Lax A. J., Barrow, P. A., Jones, P. W. and Wallis, T. S. (1995). Current perspective in Salmonellosis. *British Veterinary Journal*, 151: 351-377.

Lazazzera, B. A. (2005). Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Current Opinion in Microbiology*, 8: 222-227

Le Hello, S., Bekhit A., Granier, S. A., Barua, H., Beutlich, J., Zając, M., Münch, S., Sintchenko, V., Bouchrif, B., Fashae, K., Sontag, L., Fabre, L., Garnier, M., Guibert, V., Howard, P., Doublet, B. and François-Xavier, W. (2013a). The global establishment of a highly flouroquinolone resistant *Salmonella enterica* serotype Kentucky *ST198* strain. *Front microbiology,* 4: 395 – 399.

Le Hello, S., Harrois, D., Bouchrif, B., Sontag, L., Elhani, D. and Guibert, V. (2013b). Highly drug-resistant *Salmonella* Kentucky ST198-X1: a microbiological study. *The Lancet Infectious Disease,* 13: 672– 679.

Le Hello, S., Hendriksen, R. S., Doublet, B., Fisher, I., Nielsen, E. M., Whichard, J. M., Bouchrif, B., Fashae, K., Granier, S. A., Silva, N. J., Cloeckaert, A., Threlfall, E. J., Angulo, F. J., Aarestrup, F. M., Wain, J. and Weill, F. X. (2011) International Spread of an Epidemic; Population of *Salmonella enterica* Serotype Kentucky ST198 Resistant to Ciprofloxacin. *Journal of Infectious Diseases,* 204: 675- 684.

Lesnick, M., Reiner, N., Fierer, J. and Guiney, D. (2001). The *Salmonella* spvB virulence gene encodes an enzyme that ADP ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Molecular Microbiology,* 39: 1464-1470.

Lewandrowski, K. B. (2015). Veterinary Laboratory medicine: Small and exotic animals. *Clinic in laboratory Medicine*, 25(3): 272 – 2712.

Li, X. Z. and Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria, *Drugs*, 64:159–204.

Liu, W., Yang, Y., Chung, N. and Kwang, J. (2001). Induction of humoral immune response and protective immunity in chickens against *Salmonella* Enteritidis after a single dose of killed bacterium-loadedmicrospheres. *Avian Diseases*, 45: 797- 806.

MacLennan, C. A. (2013) Vaccines for low-income countries; review, *Seminars in Immunology*, 25: 114 – 123.

MacLennan, C. A., Gilchrist, J. J., Gordon, M. A., Cunningham, A. F., Cobbold, M., Goodall, M., Kingsley, R. A., van Oosterhout, J. J., Msefula, C. L. and Molyneux, M. E. (2010). Dysregulated humoral immunity to non typhoidal *Salmonella* in HIV-infected African adults. *Science*, 328: 508 – 512.

Maclennan, C. A., Gondwe, E. N., Msefula. L., Kingsley, R. A., Thompson, N. R., White, S. A., Goodall, M., Pickard, D. J. and Drayson, M. T. (2008). The neglected role of antibody in protection against bacteremia caused by non typhoidal strains of *Salmonella* in African children. *Journal of Clinical Investigations*, 118: 1553 – 1562.

Mah, T. F. C. and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, pp. 34-39.

Majowicz, S. E., Musto, J. and Scallan, E. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases*, 50: 882 - 889.

Mamman, P. H., Kazeem, H. M., Raji, M. A., Nok, A. J. and Kwaga, J. K. P. (2014). Isolation and Characterization of *Salmonella* Gallinarum from outbreaks of fowl typhoid in Kaduna State, Nigera. *International Journal of Public Health Epidemiology*, 3: 082 – 088.

Mandomando, I., Macete, E., Sigauque, B., Morais, L., Quinto, L., Sacarlal, J., Espasa, M., Valles, X., Bassat, Q., Aide, P., Nhampossa, T., Machevo, S., Ruiz, J., Nhacolo, A., Menendez, C., Kotloff, K. L. and Alonso, P. L. (2009). Invasive nontyphoidal *Salmonella* in Mozambican children. *Tropical Medicne and International Health*, 14:1467 – 1474.

Mankhambo, L. A., Chiwaya, K. W., Phiri, A. and Graham, S. M. (2006). Lobar pneumonia caused by non typhoidal *Salmonella* in a Malawian child. *Pediatric Infectious Disease*, 25: 1190 – 1192.

Marin, C., hernandiz, A. and Lainez, M. (2009). Biofilm development capacity of *Salmonella* strains isolated in poultry risk factors and their resistance against disinfectants. *Poultry Science*, 88: 424 – 431.

Marlovits, T. C. and Stebbins, C. E. (2010). Type III secretion systems shape up as they ship out. *Current Opinion in Microbiology*, 13: 47–52.

Marlovits, T. C., Kubori, T., Sukhan, A., Thomas, D. R., Gala'n, J. E. and Unger, V. M. (2004). Structural insights into the assembly of the type III secretion needle complex. *Science*, 306: 1040 – 1042.

Mastroeni, P., Chabalgoity, J. A. and Dunstan, S. J., (2001). *Salmonella*: Immune responses and vaccines. *Veterinary Journal*, 161: 132 – 164.

Mbuko, I. J., Raji, M. A., Ameh, J. A., Sai'du, L., Musa, W. I. and Abdu, P. A. (2009). Prevalence and seasonality of fowl typhoid disease in Zaria- Kaduna State, Nigeria. *Journal of Bacteriology Research*, 1: 001 – 005.

McHan, F., Shott, E. B. and Brown, J. (1991). Effect of feeding selected carbohydrates on the in vivo attachment of *Salmonella* Typhimurium in chick ceca. *Avian Disease*, 35: 328–331.

Medina, E. and Guzman, C. A. (2001) Use of live bacterial vaccine vectors for antigen delivery: Potential and limitations. *Vaccine*, 19: 1573 – 1580.

Meltzer, E. and Schwartz, E. (2007). Enteric fever: an Israeli perspective. *Israel Medical Association Journal*, 9(10): 736 – 741.

Metawe, Y. F. and Tulip A. A. (2004). Epidemiological Studies on *Salmonellae* in Poultry Feed Mills. *Benha Veterinary Medicine Journal*, 15: 134 - 146.

Milledge, J., Calis, J. C., Graham, S. M., Phiri, A., Wilson, L. K., Soko, D., Walsh, A. L., Rogerson, S. J., Molyneux, M. E. and Molyneux, E. (2005). Aetiology of neonatal sepsis in Blantyre, Malawi: 1996 – 2001. *Ann Tropical Pediatrics*, 25: 101 – 110.

Molbak, E. S. and Josefsen, M. H. (2016) Bacteria: *Salmonella* Non-Typhi. Encyclopedia of Food Safety. pp 501–514.

Molyneux, E. M., Mankhambo, L. A., Phiri, A., Graham, S. M., Forsyth, H., Walsh, A. L. and Wilson, L. K. (2009). The outcome of non typhoidal *Salmonella* meningitis in Malawian children, 1997 – 2006. *Ann Tropical Pediatric*. 29: 13 – 22.

Morpeth, S. C., Ramadhani, H. O. and Crump, J. A. (2009). Invasive Non Typhi *Salmonella* disease in Africa. *Clinical Infectious Disease*, 49: 606 – 611.

Mshelbwala, F. M., Ibrahim, N. D., Saidu, S. N., Azeez, A. A., Akinduti, P. A., Kwanashie, C. N., Fakilahyel, A. K., Muhammed, M., Fagbamila, I. O. and Luka, P. D. (2017). Motile Salmonella serotypes causing high mortality in poultry farms in three South-Western States of Nigeria. *Veterinary Record Open* ,4:e000247. doi:10.1136/ vetreco-2017-000247

Münch, S., Braun, P., Wernery, U., Kinne, J., Pees, M. and Flieger, A. (2012). Prevalence, serovars, phage types, and antibiotic susceptibilities of *Salmonella* strains isolated from animals in the United Arab Emirates from 1996 to 2009. *Tropical Animal Health Production*, 44: 1725–1738.

Murray, C. J., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, c., Ezzati, M., Shibuya, K., Salomon, J. A., Abdalla, S., Aboyans, V., Abraham, J. and Ackerman, I. (2012) Disability adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990 – 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, 380: 2197 – 2223.

Murugka, H. V., Rahman, H. and Dutta, P. K. (2003). Distribution of virulence genes in *Salmonella* serovars isolated from man and animals. *Indian Journal of Medical Research.* 117: 66 – 70.

Nikaido, E., Yamaguchi, A. and Nishino, K. (2008). *AcrAB* Multidrug efflux pump regulation in *Salmonella enterica* serovar Typhimurium by *RamA* in response to environmental signals. *Journal of Biological chemistry*, 283(35): 24245 – 24253.

Nordqvist, C. (2016) *Salmonella*: Symptoms, Treatment and Prevention. Article; Infectious Diseases / Bacteria / Viruses. updated 17 February.

Nourhan, N. M., Sadek, M. A. and Wafaa, R. A. (2014). Studies on pathogenecity of *Salmonella enterica* serovar Kentucky in chicken. *Egypt Journal of Comparative Pathology and Clinical Pathology*, 27(1): 18 - 25.

Nourhan, N. M., Sadek, M. A., Wafaa, R. A. A., Soliman, E. M., Eman, S. A. Z. and Ibrahim, H. M. (2015). Efficacy of locally prepared *Salmonella* Kentucky vaccine in chicken. *Benha Veterinary Medical Journal*, 29(2): 153-160.

Nye, K. J., Fallon, D. and Frodsham, D. (2002). An evaluation of the performance of XLD, DCA, MLCB and ABC agars as direct plating media for isolation of *Salmonella enterica* from faeces. *Journal of Clinical Pathology*, 55(4): 286 – 288.

OIE Terrestrial Manual, (2008). Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). 6th edition. Chapter 2.3.11 pp 514 - 526

OIE Terrestrial Manual, (2010). Salmonellosis. Chapter 2.9.9. pp 4- 5.

OIE Terrestrial Manual, (2012). Fowl typhoid and Pullorum disease. World assembly of delegates of OIE, 2: 9-11

Okoro, C. K., Kingsley, R. A., Quail, M. A., Kankwatira, A. M., Feasey, N. A., Parkhill, J., Dougan, G. and Gordon, M. A. (2012). High resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive non typhoidal *Salmonella* Typhimurim disease. *Clinical Infectious Disease*, 54: 955 – 963.

Oliveira, S. D., Rodenbusch, C. R., Michael, G. B., Cardoso, M. I. R., Canal, C. W. and Brandelli, A. (2003). Detection of virulence genes in *Salmonella* Enteritidis isolates from different sources. *Brazilian Journal of Microbiology*, 34(1): 123-124.

Osman, K. M., Moussa, I. M. I., Yousef, A. M. M., Aly, M. M., Radwan, M. I. and Alwathnani, H. A. (2010). Pathogenicity of some Avian *Salmonella* serovars in Two Different Animal Models: SPF-Chickens and BALB/c Mice. *Environment and We International Journal of Science and Technology*, 5: 65 – 78.

Oxoid (2018). X. L. D. Agar (CM0469) Oxoid microbiology products. Thermo Fisher Scientific Inc. www.oxoid.com.

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J. and Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998 – 2008. *Emerging Infectious Disease*, 19: 407 – 415.

Papezova, K., Havlickova, H., Sisak, F., Kummer, V., Faldyna, M. and Rychlik, I. (2008). Comparison of live and inactivated *Salmonella* Typhimurium vaccines containing different combinations of SPI-1 and SPI-2 antigens in poultry. *Veterinari Medicina*, 53(6): 315 – 323.

Parkhill, J., Dougan, G., James, K. D., Thomson, N. R., Pickard, D. and Wain, J. (2001). Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature*, 413: 848 – 852.

Parry, C. M., Thomas, S., Aspinall, E. J., Cooke, R. P., Rogerson, S. J., Harries, A. D. and Beeching, N. J. (2013). A retrospective study of secondary bacteremia in hospitalized adults with community acquired non typhoidal *Salmonella* gastroenteritis. *BMC Infection and Disease*, 13: 107 - 110.

Parsons, B. N., Humphrey, S., Salisbury, A. M., Mikoleit, J., Hinton, J. C., Gordon, M. A. and Wigley, P. (2013). Invasive non typhoidal *Salmonella* Typhimurium ST313 is not host restricted and have an invasive phenotype in experimentally infected chickens. *PLoS One Tropical Disease*, 7:e2487.

Pei, Y., Parreira, V. R., Roland, K. L., Curtiss, R. and Prescott, J. F. (2014). Assessment of attenuated *Salmonella* vaccine strains in controlling experimental *Salmonella* Typhimurium infection in chickens. *Canadian Journal Veterinary* Research, 78(1): 23-30.

Penha Filho, R. A. C. and Berchieri, A. J. (2012). Humoral and cellular immune response generated by different vaccine programs before and after *Salmonella* Enteritidis challenge in chickens. *Vaccine*, 30(52): 7637 – 7643.

Piddock, L. J. (2006). Multidrug-resistance efflux pumps? Not just for resistance, *National Revised Microbiology*. 4: 629 – 636.

Plotkin, S. A. (2009). Vaccines: The fourth century. *Clinical Vaccine Immunology*, 16: 1709–1719.

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms, *Ann Tropical Medical*. 39: 162–176.

Popoff, M. Y., Bockemiihl, J. and Brenner, F. W. (2000). Supplement 1998 to Kauffmann White Scheme. *Research in Microbiology*, 151: 63 – 65.

Preziosi, M. J., Kandel, S. M., Guiney, D. G. and Browne, S. H. (2012). Microbiological analysis of non typhoidal *Salmonella* strains causing distinct syndromes of bacteremia or enteritis in HIV/AIDS patients in San Diego, California. *Journal of Clinical Microbiology*, 3: 24 – 30.

Putman, M., van Veen, H. W. and Konings, W. N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiology Molecular Biology Review*, 64: 672 – 693.

Raufu, I. A., Fashae, K., Ameh, J. A., Ambali, A., Ogunsola, F. T., Coker, A. O. and Hendriksen, R. S. (2014). Persistence of fluoroquinolone-resistant *Salmonella enterica* serovar Kentucky from poultry and poultry sources in Nigeria. *Journal of Infection in Developing Countries*, 8(3): 384 - 388.

Reddy, E. A., Shaw, A. V. and Crump, J. A. (2010). Community acquired bloodstream infections in Africa: a Systematic review and meta-analysis. *Lancet Infectious* Disease, 10(6): 417 – 432.

Revolledo, L. (2012). Avian Salmonellosis, vaccines and immune mechanisms of protection: present and future perspectives. *Poultry Health and Biosecurity*, 24: 5- 9.

Rotger, R. and Casadesus, J. (1999). The virulence plasmids of *Salmonella*. *International Microbiology*, 2: 177 – 184.

Sanderson, K. E., Liu, Shu-Lin. and Johnston, R. N. (2015). *Salmonella* Typhi and *Salmonella* Paratyphi A. *Molecular Medical Microbiology*, 2(71): 1275 – 1306.

Santos, R. L., Tsolis, R. M., Bäumler, A. J. and Adams, L. G. (2003) Pathogenesis of *Salmonella* induced enteritis. *Brazilian Journal of Medical Biology Research*, 36: 3–12.

Sasai, K., Yoshimura, K. and Lillehoj, H. S. (1997). Analysis of splenic and thymic lymphocyte subpopulations in chickens infected with *Salmonella* Enteritidis. *Veterinary Immunology and Immunopathology*, 59: 359–367.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., and Roy, S. L. (2011). Food borne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases*, 17(1): 7-15.

Schmidt, H. and Hensel, M. (2004) Pathogenicity islands in bacterial pathogenesis. *Clinical Microbiology Review*, 17: 14–56.

Shah, A. H and Korejo, N. A. (2012). Antimicrobial Resistance Profile of *Salmonella* Serovars Isolated from Chicken Meat. *Journal of Veterinary and Animal Science*, 2: 40 – 46.

Shemesh, M., Tam, A. and Steinberg, D. (2007). Differential gene expressing profiling of *Streptococcus* mutants cultured under biofilm and planktonic conditions. *Microbiology*, 153: 1307 – 1317.

Shivaprasad, H. L. (2000). Fowl typhoid and pullorum disease. *Revised Science Techniques.* 19: 405 – 424.

Siggins, M. K., O'Shaughnessy, C. M., Pravin, J., Cunningham, A. F., Handerson, I. R., Drayson, M. T. and MacLennan, C. A. (2014). Differential timing of antibody mediated phagocytosis and cell-free killing of invasive African *Salmonella* allows immune evasion. *Europe Journal of Immunology*, 44: 1093 – 1098.

Snow, L. C., Davies, R. H., Christiansen, K. H., Carrique-Mas, J. J., Cook, A. J. C. and Evans, S. J. (2010). Investigation of risk factors for *Salmonella* on commercial egg-laying farms in Great Britain, 2004 - 2005. *Veterinary Record*, 166: 579-586.

Sommet, J., Missud, F., Holvoet. L., Ithier, G., Lorrot, M., Benkerrou, M. and Faye, A. (2013). Morbidity among child travellers with sickle cell disease visiting tropical areas: an observational study in a French tertiary care center. *Arch Disease of Children*, 98: 533 – 536.

Song, J., Gao, X. and Galan, J. E. (2013). Structure and function of *Salmonella* Typhi chimaeric A2B5 Typhoid toxin. *Nature*, 499: 350 – 354.

Soria, M. A., Bonnet, M. A. and Bueno, D. J. (2015) Relationship of *Salmonella* infection and inflammatory intestinal response with hematological and serum biochemical values in laying hens. *Journal of Veterinary Immunology and Immunopatholog*, 165(3): 145–153.

Switt, M. I. A. (2017). *Salmonella* Kentucky. ed Weller, L. D. (2017) *Salmonella enterica* serovar Kentucky strain SRC73. *Food safety*, pp 1 – 3.

Talbert, A. W., Mwaniki, M., Mwarumba, S., Newton, C. R. and Berkley, J. A. (2010). Invasive bacterial infections in neonates and young infants born outside hospital admitted to a rural hospital in Kenya. *Paediatrics Infection and Disease Journal*, 29: 954 – 949.

Teplitski, M., Al-Agely, A. and Ahmer, B. M. M. (2006) Contribution of the SirA regulon to biofilm formation in *Salmonella enterica* serovar Typhimurium. *Microbiology*, 152: 3411–3423.

Terzolo, H. R. and Chacana, P. A. (2016) Fowl Tyhoid in Laying Hens: Description of the disease and Protection Conferred by a Live *Salmonella* Enteritidis Vaccine. pp 1-9

The Oxoid Manual (2006). 9th edition Oxoid Limited. Wade road, Basingstoke, Hampshire, England.

Tizard, I. R. (2004). Veterinary Immunology, an Introduction. 7th ed. Philadelphia: Saunders; pp. 27–31.

Townsend, S. M., Kramer, N. E., Edwards, R., Baker, S., Hamlin, N., Simminds, M., Stevens, K., Maloy, S., Parkhill, J., Dougan, G. and Baumler, A. J. (2001). *Salmonella enterica* serovar Typhi posseses a unique repertoire of fimbrial gene sequences. *Infection and Immunology*, 69: 2894 – 2901.

Trebicka, E., Shanmugam, N. K., Mikhailova, A., Alter, G. and Cherayil, B. J. (2014). Effect of human immunodeficiency virus infection on plasma bactericidal activity against *Salmonella enterica* serovar Typhimurium. *Clinical Vaccine Immunology*, 21: 1437 – 1442.

Turki, Y., Ouzari, H., Mehri, I., Ben Aissa, R. and Hassen, A. (2012). Biofilm formation, virulence gene and multidrug resistance in *Salmonella* Kentucky isolated in Tunisia. *Food Research International*, 45: 940 – 946.

Uematsu, S., Jang, M. H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., Kato, H., Sougawa, N., Matsui, H., Kuwata, H., Hemmi, H. and Akira, S. (2006). Detection of pathogenic intestinal bacteria by toll-like receptor 5 on intestinal cd11c+ lamina propria cells. *Nature Immunology*, 7: 868-874.

Useh, N. M., Ngbede, E. O., Akange, N., Thomas, M., Foley, A., Keena, M. C., Nelson, E., Christopher-Hennings, J., Tomita, M., Suzuki, H. and Scaria, J. (2016). Draft Genome Sequences of 37 *Salmonella enterica* Strains Isolated from Poultry Sources in Nigeria. *Genome announcement*, 4(3): 315 – 316.

Van Immerseel, F. (2016). Immunity conferred by live *Salmonella* vaccine strains in chickens and their mode of action. *Salmonella 360^0 Bulletin*, Review, pp: 1-3.

Vernikos, G. S. and Parkhill, J. (2006) Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the *Salmonella* pathogenicity islands. *Bioinformatics*, 22: 2196–2203.

Waltman, W. D., Gast, R. K. and Mallinson, E. T. (1998). Salmonellosis: A laboratory Manual for the Isolation and Identification of Avian Pathogens. American Association of Avian Pathologist 4th Edition, Swayne *et al* (eds) university Of Pennsylvania USA, 4 – 13.

Wang, S., Li, Y. and Scarpellini, G. (2010). *Salmonella* vaccine vectors displaying delayed antigen synthesis in vivo to enhance immunogenicity. *Infectious Immunology*, 78: 3969–3980.

Wasyl, D. and Hoszowski, A. (2012). First isolation of ESBL-producing *Salmonella* and emergence of multiresistant *Salmonella* Kentucky in turkey in Poland. Food Research Institute, 45: 958–961

Weill, F. X., Bertrand, S., Guesnier, F., Baucheron, S., Cloeckaert, A. and Grimont, P. A. (2006). Ciprofloxacin-resistant *Salmonella* Kentucky in travellers. *Emerging Infectious Disease*, 12: 1611–1612.

White, A. P., Gibson, D. L., Collinson, S. K., Banser, P. A. and Kay, W. W. (2003). Extracellular polysaccharides associated with thin aggregative fimbriae of *Salmonella enterica* serovar Enteritidis. *Journal of Bacteriology*, 185: 5398 – 5407.

Wong, K. K., McClelland, M., Stillwell, L. C., Sisk, E. C., Thurston, S. J. and Saffer, J. D. (1998). Identification and sequence analysis of a 27-kilobase chromosomal fragment containing a *Salmonella* pathogenicity island located at 92 minutes on the chromosome map of *Salmonella enterica* serovar Typhimurium. *Infection and Immunology*, 66: 3365–3371.

Yeasmin, (2010). On farm investigation on the efficacy of *Salmonella* vaccine prepared at BAU. *MS thesis*, submitted to the Department of Microbiology and Hygiene, BAU, Mymensingh.

Yoon, H., Ansong, C., McDermott, J. E., Gritsenko, M., Smith, R. D. and Heffron, F. (2011). Systems analysis of multiple regulator perturbations allowa discovery of virulence factors in *Salmonella. BMC Systemic Biology*, 5: 100 – 105.

Yoon, H., McDermott, J. E., Porwollik, S., McClelland, M. and Heffron, F. (2009). Coordinated regulation of virulence during systemic infection of *Salmonella enterica* serovar Typhimurium. *PLOS Pathology*, 5: 100 – 306.

Zhang-Baeber, L., Turner, A. K. and Barrow, P. A. (1999). Vaccination for the Control of *Salmonella* in Poultry. *Vaccine*. 17: 2538 – 2545.

APPENDICES

Appendix I: Prepared experimental vaccines



Vials contain live (5-bromouracil attenuated) vaccine, administered at 0.5 mL/birds subcutaneously

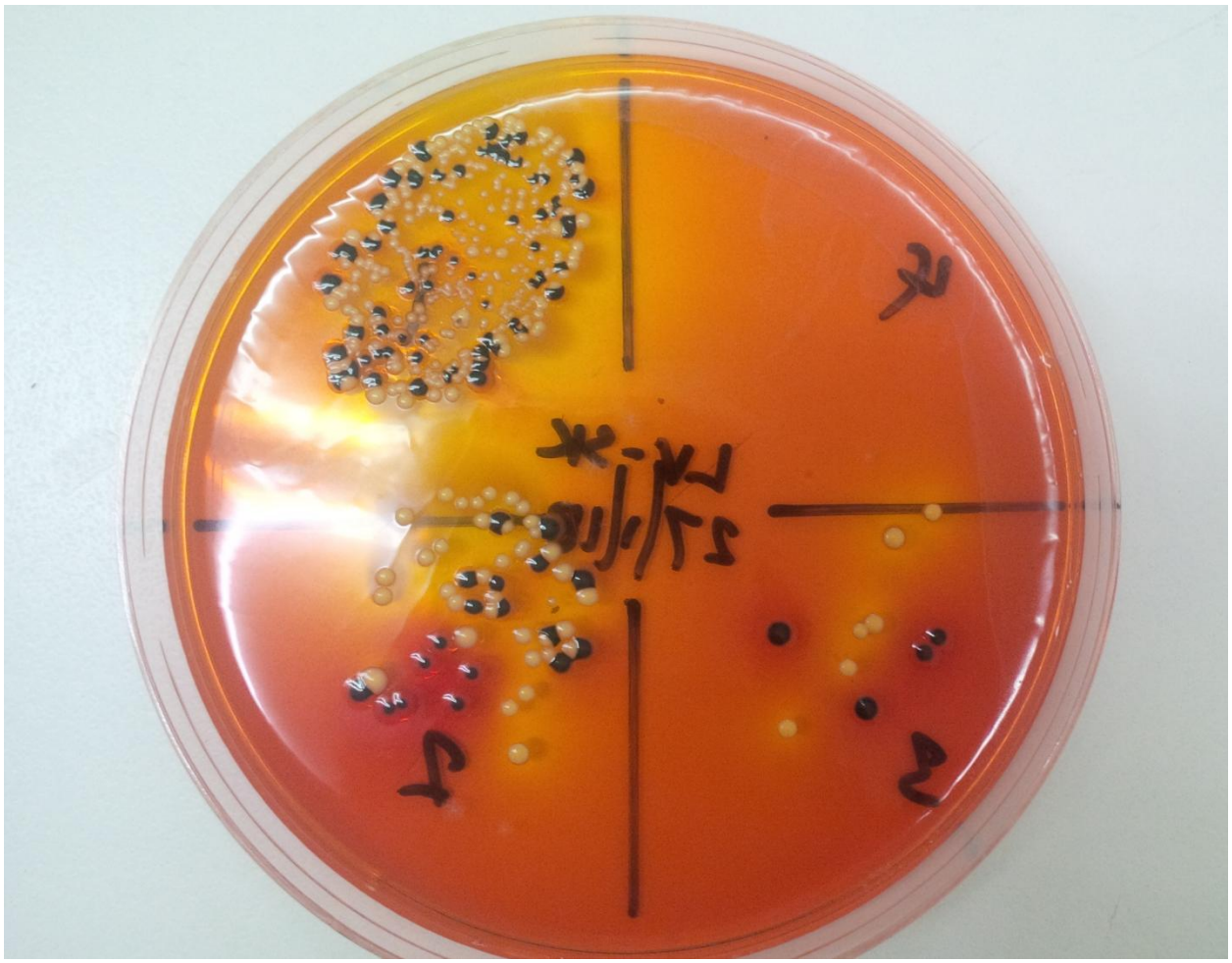
Bottles (200 mL) contain formalin inactivated vaccine, administered at1 mL/bird subcutaneously

Appendix II: Typical colony in faecal swab of broilers on XLD agar prior to challenge at day 32 of age



The yellow colonies could be *Citrobacter* spp, *Escherichia coli* or *Proteus* spp; they are capable of fermenting Xylose to causing an acid pH in the medium therefore forming yellow colonies.

Appendix III: Typical colonies in faecal swab of broilers on XLD agar after challenge at day 32 of age



Black colonies are Salmonellae. Yellow colonies could be *Citrobacter* spp, *Escherichia* coli or *Proteus* spp.

Appendix IV: Result showing Mean ± Standard Error (S.E.M) of *Salmonella* Kentucky in CFU/mL to (Log_{10} N) re-isolated from faeces after challenge

	Mean (Log_{10} N) ± S.E.M			
Group	**Day 35**	**Day 42**	**Day 49**	**Day 54**
Live vaccine	3.53 ± 0.32	2.99 ± 0.19	2.52 ± 0.09	0 ± 0
Inactivated vaccine	3.88 ± 0.14	3.28 ± 0.20	2.66 ± 0.08	2.54 ± 0.06
Unvaccinated	5.54 ± 0.20	6.13 ± 0.27	6.63 ± 0.46	5.04 ± 0.17

Appendix V: Result of ELISA showing the mean number (Log_{10} ng/mL) of developed antibody (IgY) titre in serum of experimental birds

	Age of birds							
Group	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**	**Day 42**	**Day 49**	**Day 54**
Live vaccine	0.43	0.64	1.02	1.30	1.35	1.36	1.34	1.31
Killed vaccine	0.39	0.65	1.05	1.26	1.32	1.35	1.32	1.28
Unvaccinated	0.33	0.58	0.49	0.56	0.92	1.06	0.93	0.94

Appendix VI: Preparation of Nutrient broth (Oxoid, UK)

Ingredients	Grams/litre
Lab-lemco powder	1
Yeast extract	2
Peptone	5
Sodium chloride	5

Suspend 13 g of powder in 1 litre of distilled water, mix well and dispense into Schott Duran bottles. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Appendix VII: Sabouraud Dextrose Agar (Oxoid, UK)

Ingredients	Grams/litre
Mycological peptone	10
Dextrose	40
Agar no. 1	15

Suspend 65 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Appendix VIII: 2 % Buffered peptone water (Oxoid, UK)

Ingredients	Grams/litre
Peptone	10
Sodium chloride	5
Disodium phosphate,	3.5
Potassium dihydrogen phosphate	1.5

Suspend 20 g in 1 litre of distilled water, dissolve completely and dispense into Schott Duran bottles. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Appendix IX: Preparation of Citrate buffer and Phosphate buffer

Citrate Buffer

800 ml of distilled water was prepared in graduated Schott Duran bottle, add 12.044 g of sodium citrate dehydrate, add 11.341 g of citric acid monohydrate, adjust to reqired pH using 0.1 N HCl, make up total volume to 1 litre using distilled water. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Phosphate Buffer

Suspend the following in 1 litre of distilled water, 8 g of NaCl, 1.15 g of Na_2HPO_4, 200 mg of KH_2PO_4 and 200 mg of KCl. Dissolve completely and dispense to Schott Duran bottles. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Appendix X: Chicken IgY (Immunoglobulin Y) Enzyme Linked Immunosorbent Assay (ELISA) kit procedures

Aspirate all wells, and wash plate three times with 350 µL wash buffer (soaking for 1 to 2 minutes for each wash). After the third wash, invert plate, and clap the plate on absorbent filter papers or other absorbent material.

Sample dilution

1 µL of serum sample was diluted in 99 µL of Standard Dilution Buffe**r**

Reagent preparation and storage

Wash Buffer: 30 ml concentrated Wash Buffer was diluted into 750 mL distilled water. Unused solution is kept at 4^{o}C. If crystals have formed in the concentrate, it is warmed in 40 ^{o}C in water bath and mixed gently until the crystals have dissolved completely. Solution should be at room temperature (25 ^{o}C) before use.

Standard: 100 ng/mL of standard solution; 1 mL sample/ standard dilution buffer into one standard tube, keep the tube at room temperature (25 ^{o}C) for 10 minutes and mix them thoroughly. Label 6 Eppendorf tubes with 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.563 ng/mL, respectively. Add 0.3 mL of the sample/standard dilution buffer into each tube. Add 0.3 mL of the above 100 ng/mL standard solution into 1st tube and mix them thoroughly. Transfer 0.3 mL from the 1st tube to 2nd tube and mix them thoroughly. Transfer 0.3 mL from 2nd tube to 3rd tube and mix them thoroughly, and so on.

Note: It is best to use standard Solutions within 2 hours. The standard Solution shall be at 4 ^{o}C up to 12 hours. Or store it at - 20 ^{o}C up to 48 hours. Avoid repeated freeze-thaw cycles.

Preparation of Biotin-labeled Antibody Working Solution

Prepare it within 1 hour before experiment.

Calculate required total volume of the working solution: 0.1 mL / well by quantity of wells. (Allow 0.1 – 0.2 mL more than the total volume). Dilute the Biotin-detection antibody with Antibody Dilution Buffer at 1: 100 and mix them thoroughly. (i.e Add 1 μL Biotin-labeled antibody into 99 μL Antibody Dilution Buffer.)

Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution

Prepare it within 30 minutes before experiment.

Calculate required total volume of the working solution: 0.1 mL / well x quantity of wells. (Allow 0.1 mL – 0.2 mL more than the total volume). Dilute the SABC with SABC Dilution Buffer at 1: 100 and mix them thoroughly. (i.e Add 1 μL of SABC into 99 μL SABC Dilution Buffer.)

Assay Procedure

1. Set standard, test sample and control (Zero) wells on the pre-coated plate respectively and record their positions.
2. Aliquot 0.1 mL of 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.563 ng/mL, standard solutions into the standard wells.
3. Add 0.1 mL Sample/ standard Dilution Buffer into control(zero) well
4. Add 0.1 mL of diluted serum sample into test sample wells
5. Seal the plate with a cover and incubate at 37 ^{o}C for 90 minutes.

6. Remove the cover and discard the plate content, and wash plate 2 times with wash buffer. Do not let the wells dry completely at any time
7. Add 0.1 mL Biotin-labeled antibody working solution into above wells (Standard, test sample and zero wells). Add the solution at the bottom of each well without touching the side wall.
8. Seal the plate with a cover and incubate at 37 ^{o}C for 60 minutes.
9. Remove the cover, and wash plate 3 times with Wash Buffer, and let the wash buffer stay in the wells for 1 minute each time.
10. Add 0.1 mL of SABC Working Solution into each well, cover the plate and incubate at 37 ^{o}C for 30 minutes.
11. Remove the cover and wash plate 5 times with Wash Buffer, and let the wash buffer stay in the wells for 1 -2 minute each time.
12. Add 90 μL TMB Substrate into each well, cover the plate and incubate at 37 ^{o}C in dark within 15 – 30 minutes. It will turn blue in the first 3 – 4 wells (with most concentrated IgY standard solutions), the other wells may not display obvious colour.
13. Add 50 μL Stop Solution into each well and mix them thoroughly. The colour changes to yellow immediately.
14. Read the O.D. absorbance at 450 nm in Microplate reader immediately after adding the stop solution.

The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) versus the respective concentration of the Standard solution (X). The IgY concentration of the samples can be interpolated from the standard curve.