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CHARACTERIZATION OF *S.* GALLINARUM, *S.* PULLUROM AND *S.* ENTERITIDIS ISOLATED FROM CHICKEN IN CENTRAL ETHIOPIA AND EVALUATION OF PROTECTIVE AND CROSS PROTECTIVE EFFICACY OF SALMONELLA GALLINARUM 9R VACCINE AGAINST *S.* GALLINARUM, *S.* PULLUROM AND *S.* ENTERITIDIS

MSc Thesis

By

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June, 2015

Bishoftu, Ethiopia

Characterization of S. Gallinarum, S. Pullurom and S. Enteritidis isolated from chicken in central Ethiopia and Evaluation of Protective and Cross protective efficacy of Salmonella Gallinarum 9R vaccine against S. Gallinarum S. Pullurom and S. Enteritidis

A thesis submitted to College of Veterinary Medicine and Agriculture Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology

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As members of the Examining Board of the final MSc *Open Defense*, we certify that we have read, evaluated the thesis prepared by Kassaye Adamu Reda titled " Characterization of S. Gallinarum, S. Pullurom and S. Enteritidis isolated from chicken in central Ethiopia and Evaluation of Protective and Cross protective efficacy of Salmonella Gallinarum 9R vaccine against S. Gallinarum S. Pullurom and S. Enteritidis" and recommended that the thesis it be accepted as fulfilling the thesis requirement for the Degree of Master of Science in Veterinary microbiology.

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DEDICATION

I dedicate this manuscript to my mother and my father who passed away without seeing any of my achievements.

STATEMENT OF AUTHOR

First, I declare that this thesis is my bonafide work and that all sources of material used for this thesis been duly acknowledged. This thesis is submitted for partial fulfillment of the requirements of an advanced (MSc) degree at Addis Ababa University College of Veterinary medicine and Agriculture and provide my consent that it can be deposited at the university/college library to be made available to users. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

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LIST OF ABBREVIATION

BGA	Brilliant Green Agar
bp	base pair
BWD	Bacillary White Diarrhea
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Unit
CFU	Colony forming unit
CSA	Central Statistics Agency
DAMPs	Danger-Associated Molecular Pattern Molecules
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immune Sorbent Assay
FT	Fowl typhoid
GALT	Gut Associated Lymphoid Tissue
LPS	Lipopolysaccharides
MRVP	Methyl red- voges-proskauer
NLRs	NOD-like receptors
NMSA	National Metrological Service Agency
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PD	Pullorum disease
PRRs	Pattern-recognition receptors
RSPAT	Rapid Serum Plate Agglutination test
S/C	Subcutaneous injection
SE	*Salmonella* Enteritidis
SG9R	*Salmonella* Gallinarum 9R vaccine strain
SP	*Salmonella* Pullorum
SPF	Specific pathogen free
SPI	Salmonella Pathogenic Islands
SSA	Salmonella Shigella Agar
T3SS	Type Three Secretion System
TAE	Tris-acetate-EDTA
TSI	Triple sugar Iron agar slant
XLD	Xylose Lysine Desoxycholate agar

Abstract:- Salmonellosis is one of the most important bacterial diseases of poultry with significant impact due to the loss of productivity in affected birds and its potential public health hazard. This study is performed with the aim of evaluating the immunizing and protective efficacy of the vaccine currently being used against *salmonella* strains precipitating disease outbreaks in poultry. Necropsy samples were collected from suspected cases showing typical pathological lesions for bacterial isolation. Isolation and identification of bacteria was carried out employing conventional bacteriological and biochemical methods followed by characterization using mPCR. *Salmonella* strains (12 *Salmonella* Gallinarum, 1 *Salmonella* Pullorum and 1 *Salmonella* Enteritidis) isolated from suspected cases were used for the challenge experiment in chicken vaccinated against salmonellosis at 42 days of age using 9R vaccine. Vaccinated chicken showing strong immune reaction as observed with slide agglutination test were challenged with field strains of virulent *salmonella* Gallinarum, *salmonella* Pullorum and *salmonella* Enteritidis strains (5×10^7 CFU/ml of each) 1ml orally at 14 days post vaccination. Post challenge follow-up for 2 weeks to determine the degree of protection of the vaccine showed that no mortality was observed in the vaccinated and *salmonella* Gallinarum challenged group despite that 3 (20%) chicken had pinpoint hemorrhages on the liver and enlarged spleen during post-mortem examination. However, in the non-vaccinated group, 8(53.3%) birds died, 5(33.3%) showed macroscopic lesions while the remaining 2(13.3%) had no any lesion. Observation of mortality and extent of lesion after challenge with the *salmonella* Pullorum virulent strain, showed no mortality in the immunized group while 2 (13.3%) birds died in the un-immunized group. One (6.7%) chick in the immunized group showed macroscopic lesion, while 13(86.7%) chicken showed post-mortem lesion in the un-immunized group. In *salmonella* Enteritides virulent strain challenge group, no mortality and macroscopic lesion were observed in vaccinated group, while postmortem lesion were observed with no mortality in 10(66.7%) chicken in the non vaccinated group. Assessment of the degree of protection showed that vaccination with live *salmonella* Gallinarum 9R strain significantly reduced the mortality and severity of infection due to *salmonella* Gallinarum and also provided remarkable cross-protection against *salmonella* Pullorum and *salmonella* Enteritidis challenge in tested birds.

Key words : *Salmonella*, post-mortem lesion, protective efficacy, challenge experiment

1. INTRODUCTION

Salmonellae are facultative anaerobic Gram-negative rod shaped bacteria generally 2–5 microns long by 0.5–1.5 microns wide and motile by peritrichous flagella. Genome sizes of *Salmonella* vary among serovars ranging from 4460 to 4857 kb. *Salmonellae* belong to the family Enterobacteriaceae and are medically important pathogen for both humans and animals. The genus *salmonella* comprises a complex group of bacteria consisting of two species and six subspecies with more than 2,579 serovars (Malorny *et al.*, 2011). Two species are currently recognized in the genus *Salmonella* are *S. enterica* and *S. bongori* (Tindall, 2005). *S. enterica* can be subdivided into the subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* based on biochemical and genomic modifications (Brenner *et al*., 2000).

The majority of *Salmonellae* are lactose non fermenters, hydrogen sulfide producers, oxidase negative, and catalase positive. Other biochemical properties that allow identification of *Salmonella* include the ability to grow on citrate as a sole carbon source, decarboxylate lysine, and hydrolyze urea (Abulreesh, 2012).

Salmonellosis is one of the most important bacterial diseases in poultry industry causing heavy economic loss through mortality and reduced productivity. The disease is most significant because the causal agents of the disease are transmitted vertically from parents to offsprings (Freeman, 1985). There are mainly two serotypes of *Salmonella* species namely *Salmonella enterica* subspecies *enterica* serovar *Gallinarum* biovar Gallinarum and *Salmonella enterica* subspecies *enterica* serovar *Gallinarum* biovar Pullorum that cause fowl typhoid and pullorum disease, respectively. These two species of *Salmonella* are very important in poultry health because they are responsible for massive destruction. *Salmonellae* may cause varieties of clinical signs from acute systemic disease and gastrointestinal symptoms in the poultry flocks to embryonic problem in hatchery (Gast, 1997). Pullorum disease also known as bacillary white diarrhea (BWD) caused by *S.* Pullorum is usually confined to the first 2-3 weeks of age with occasional occurrence in adults (Shivaprashad, 1997). Fowl typhoid caused by *S.* Gallinarum is frequently referred to as a disease of adult birds but there are also reports of high mortality in young indistinguishable from those associated with pullorum diseases (Threlfall and Frost,

1990). With great expansion of poultry rearing and farming, pullorum disease and fowl typhoid have become widespread problem in Ethiopia like other countries of the world **(**Dereje, 2002**)** causing heavy economic losses in broiler, layer and breeding flocks. The loss in both broiler and layer flocks is due to morbidity, mortality, reduced production and chick quality. Mortality may vary from negligible to 10% and may reach to 80% or higher in severe outbreaks (Kleven and Yoder, 1998). The major emphasis for preventing infections is to avoid the introduction of pathogens into poultry farms through increased bio-security along with vaccination. Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (Silva *et al.*, 1981), and may stimulate the production of transient antibodies. The vaccine available in Ethiopia against the diseases is a live vaccine which is based on *Salmonella* Gallinarum 9R strain. This necessitates monitoring the safety and protective efficacy of the vaccine prior for field use against outbreaks.

The present work is therefore, undertaken with the objectives of:

- ✓ Isolation and characterization of *salmonella* from postmortem lesions during outbreaks of salmonellosis using conventional biochemical tests and molecular method.
- ✓ Evaluation of the immunizing and protective efficacy (cross protective effect) of live *Salmonella* Gallinarum 9R vaccine in chicken experimentally challenged with field strains of *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Entritidis isolated from disease outbreaks.

2. REVIEW OF LITERATURE

2.1. Historical Background

Salmonellae are widely distributed in nature. The main reservoir of these bacteria is the intestinal tract of men and warm-and cold-blooded animals **(**Threlfall, 2000)**,** except for fish, mollusks and crustaceans, which may get contaminated after being fished. Among warm-blooded animals, chickens, geese, turkeys and ducks are the most important reservoirs. Domestic animals, such as dogs, cats, turtles and birds may be carriers, and cause great risk, mainly to kids (Sharma and Katock, 1996).

The natural habitat of *salmonella* may be divided into three categories based on the specificity of the host and clinical pattern of the disease: highly adapted to men: *Salmonella* Typhi and *Salmonella* Paratyphi A, B and C, agents of typhoid fever; highly adapted to animals: *Salmonella* Dublin (bovines), *Salmonella* Choleraesuis and *Salmonella* Typhisuis (swine), *Salmonella* Pullorum and *Salmonella* Gallinarum (birds), responsible for animal paratyphoid. The third category includes most of the serovars that affect men and animals, called zoonotic *Salmonella*, responsible for worldwide-distributed foodborne diseases, and detected in most species of animals used for human consumption, wild and domestic animals (Gantois *et al*., 2009).

Salmonellae are short gram-negative bacilli, aerobic or facultative anaerobic, catalase positive, oxidase negative; they ferment sugars with gas production, produce H_2S, are nonsporogenic, and are normally motile with peritricheal flagella, except for *Salmonella* Pullorum and *Salmonella* Gallinarum, which are non motile. Optimal pH for multiplication is around 7.0; pH values above 9.0 or below 4.0 are bactericidal. Ideal temperature is between 35 to 37°C, with minimum of 5°C and maximum of 47°C. As for salt concentration, *Salmonellae* do not survive concentrations over 9% (Forshell and Wierup, 2006).

The first bacteria in the genus *Salmonella* were identified towards the end of the 19th century. *Salmonella* Typhi, the first to be recognized as a pathogen, was found in spleen and lymph nodes of humans in 1880. However, isolation and morphological description were only carried out by Gaffky, in 1884. In 1885, Salmon and Smith isolated a bacillus from diseased pigs, and called it *Bacterium* Suipestifer. They wrongly considered it the agent of swine fever. This bacterium was

later on called *Salmonella* Cholerasuis. In 1888, there was a report on *Salmonella* Enteritidis by Gaetner; in 1889, Klein identified fowl typhoid in adult birds in England, and in 1892, Loefer isolated *Salmonella* Typhimurium. In 1899, Rettger described pullorosis and differentiated it from the disease that affected pigs. In 1913, Jones used an agglutination test to identify carriers of *Salmonella* Pullorum **(**Guard-Petter, 1998).

2.2. Taxonomy and nomenclature

The genus *Salmonella* started to be classified in 1925, with the use of serological methods. S*almonella* Typhimurium, created by Loeffler (1892), and S*almonella* Paratyphi, created by Schottimuller (1899), were included in the genus. Later on, several S*almonella* serotypes were described, and classified according to White (1829) **(**Popoff *et al*., 1996) presented a proposal for the reclassification of the genus *Salmonella*, which would have two species: *Salmonella enterica* and *Salmonella bongori.* In the current classification of the Bergey's manual, all *Salmonella* serotypes belong to one of two species: *Salmonella bongori*, which has at least 10 extremely rare serotypes; and *salmonella enterica,* which is phenotypically and genotypically divided into six subspecies *enterica, salamae, arizonae, diarizonae, houtenae* and *indica*, differentiated by their biochemical behavior, mainly in terms of sugar and amino acid metabolism (Forshell and Wierup, 2006).

In the current nomenclature, the name of the serovar begins with an uppercase letter, but it is never written in italics. For example in subspecies enterica: *Salmonella enterica* subespecies *enterica* serovar Typhimurium. The short form would be *Salmonella* ser. Typhimurium or *Salmonella* Typhimurium. Other subspecies are designated by the name of the serovar, followed by its antigenic formula. Typification of *Salmonella* spp. serovars is based on the antigens found in bacterial cells, somatic (O), flagellar (H) and capsular (Vi) (Selander.,1996). Vi antigen is associated with virulence, and is only expressed by serovars Typhi, Paratyphi C and Dublin (Rycroft, 2000; Grimont *et al*., 2000). H antigen is thermolabile, whereas O and Vi are thermoresistant, and not destroyed by heating at 100°C for two hours. Somatic (O) and flagellar (H) antigens, determine different serovars in each subspecies, in a total of 2,610 serovars today, as recognized by Kauffman-White scheme (Grimont & Weill, 2007). Although all of them are

considered to be potentially pathogenic to men, only 200 are more frequently related with human disease (Baird-Parker, 1990).

Distribution according to species and subspecies is as follows: *Salmonella enterica* subsp. *enterica* (1,547 serovars); *Salmonella enterica* subsp *salamae* (513); *Salmonella enterica* subsp *arizonae* (100); *Salmonella enterica* subsp *diarizonae* (341); *Salmonella enterica* subsp *houtenae* (73); *Salmonella enterica* subsp *indica* (13); *Salmonella bongori* (23); the newly proposed species *Salmonella* subterranea was not recognized, and is considered a serovar of the bongori species (Woo, 2005).

Serovars may be further subdivided into biotypes and phagotypes. Biotyping uses different sugar fermentation patterns and assimilation of amino acids among strains of the same serovar, whereas phagotyping is based on the difference in strain susceptibility to a series of bacteriophages (Grimont *et al*., 2000). As for their antigenic profile, *salmonella* has an antigen common to all species in the Enterobacteriaceae family, called Kunin antigen. The presence of this antigen is not routinely analyzed, once it is not a relevant criterion for the differentiation between genus and species. Some serovars produce a superficial polysaccharide or capsular antigen called "Vi". It is found outside the cell wall and prevents detection of the somatic antigen. It is usually found in strains of *Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin. Vi antigens are thermolabile, and may be destroyed by heating at 100^{o}C for 10-15 minutes. The somatic antigen or "O" (Ohne), on the other hand, is specific. It is a lipopolysaccharide, and is resistant to heat and alcohol. It is made up of three parts: a lipid portion, responsible for toxicity and pyrogenic characteristics; a core portion; and the polysaccharide, which confers stability to smooth (S) variants. The "O" antigen is made up of repetitive chains with a definite spatial arrangement. The specificity of "O" antigen is given by this definite nature and the type of bond. The synthesis of this antigen is encoded by about 20 genes (locus *rfb*). Many somatic antigen factors are recognized and used in the serological identification of *salmonella*. Flagellar antigens or "H" (Hauch) antigens, are made of a protein called flagellin. Antigenic differences are related to variations in the primary structure or amino acid content of different flagellin molecules. The "H" antigen is thermolabile, may be destroyed at 100^{o}C for 10 minutes, and by slow action of alcohol 50%; but it is resistant to formaldehyde 0.5%. Agglutination of flagellar antigen forms large clumps that are quickly dissociated by

stirring. Compared with somatic agglutination, it occurs faster due to the large number of flagella in the cell, and because bacterial cells bind to each other. Spatial arrangement and intrinsic characteristics of the genus lead to the production of two different types of flagella. In most *salmonella* isolates, two genes encode flagellar antigens: *fliC* (>50 different alleles), with highly conserved terminal sequences in the genus and which encodes phase 1 antigens; and *fljB* (±30 alleles), also conserved in the genus, which encodes phase 2 antigens. These genes are expressed by a phase-variation mechanism, with *fliC* being found in all *salmonellae*, and having a homologous gene found in *E. coli;* whereas *fljB* is located in a region exclusive to the *salmonella* genome, and is found in four of the six subspecies. In some cases, triphasic strains may be isolated. Besides the other two genes, it was described that these strains presented the flagellin gene (*flpA*) in a plasmid. The genes that encode flagellin in *salmonella* spp. are generally highly conserved in extremities 5' and 3', whereas the central region is highly variable (Dunkley *et al*., 2009).

Salmonella are often classified according to the adaptation to animal hosts. A few serotypes have a limited host-spectrum (affect only one or a few animal species) such as *Salmonella* Typhi in primates, *Salmonella* Pullorum in poultry, *Salmonella* Choleraesuis in pigs, and *Salmonella* Dublin in cattle. These serotypes are relatively host specific. When these stains cause disease in humans, it is often invasive and can be life threatening. In contrast, most serotypes have a broad host-spectrum such as *Salmonella* Enteriditis and *Salmonella* Typhimurium. Typically the infection with such strains in humans cause uncomplicated gastroenteritis and does not need treatment. However the syndrome can be severe in the young and elderly and patients with weakened immunity. The *salmonella* serotypes of importance in domestic animal and the consequences of infection are indicated in Table 1 (Quinn and Markey 2003).

Table 1- *Salmonella* serotypes of clinical importance and the consequences of infection.

Salmonella	**Serotype**	**Hosts**	**Consequences of infection**
Salmonella	Typimurium	Many animal species	Enterocolitis and septicaemia
		Humans	Food poisoning
Salmonella	Dublin	Cattle	Many disease conditions
		Sheep, horses, dogs	Enterocolitis and septicaemia
Salmonella	Choleraesuis	Pigs	Enterocolitis and septicaemia
Salmonella	Pullorum	Chicks	Pullorum disease (bacillary white diarrhoea)
Salmonella	Gallinarum	Adult birds	Fowl typhoid
Salmonella	Arizonae	Turkeys	Arizona or paracolon infection
Salmonella	Enteritidis	Poultry	Often subclinical in poultry
		Many other species	Clinical disease in mammals
		Humans	Food poisoning
Salmonella	Brandenburg	Sheep	Abortion

2.3. *Salmonella* Serotypes in Poultry

2.3.1. Host-Specific *Salmonella*

Salmonella Gallinarum and *Salmonella* Pullorum cause severe disease and death of birds compared with other known *salmonella* serotypes. Chadfield *et al* suggested that *salmonella* Gallinarum invades the bursa, but the process is not time dependent, and they demonstrated no selectivity for a potential port of entry for the host-specific serotype. Avian systemic salmonellosis has 3 phases: invasion, systemic infection, and the resolution of the infection (Sadeyen *et al*., 2004). The third phase can have three results: the clearance of the bacteria, death of the birds resulting from infection, and partial clearance of the bacteria, which leads to a subclinical carrier state. The biology of pullorosis is markedly different when compared with fowl typhoid, which causes high mortality. Pullorosis induces an increase in *salmonella* in the spleen, resulting in an infection of the reproductive tract (Wigley *et al*., 2005).

2.3.2. Non-Host-Specific *Salmonella*

Salmonella infects poultry and other animals by the oral route. Non-host-specific *salmonella* in poultry is frequently involved in food poisoning in humans. In chickens, it produces systemic disease in some special cases, such as during the laying period, in chicks in the first 2 weeks of life, or after viral diseases. *Salmonellae* are not native members of the gut microbiota, but young chicks are readily colonized, and the organisms may persist in the host for some weeks or during the entire rearing period (Revolledo *et al.*, 2006). They become localized in the cecal tonsils and can occur in the upper part of the small intestine and in the gizzard and proventriculus (Fanelli *et al.*, 1971). Because most birds infected with *salmonellae* become symptomless carriers, they constitute a reservoir of the organisms, which is a potential human health hazard. Additionally, by contaminating the environment, these birds are responsible for increasing the number of infected individuals (Revolledo *et al.*, 2006).

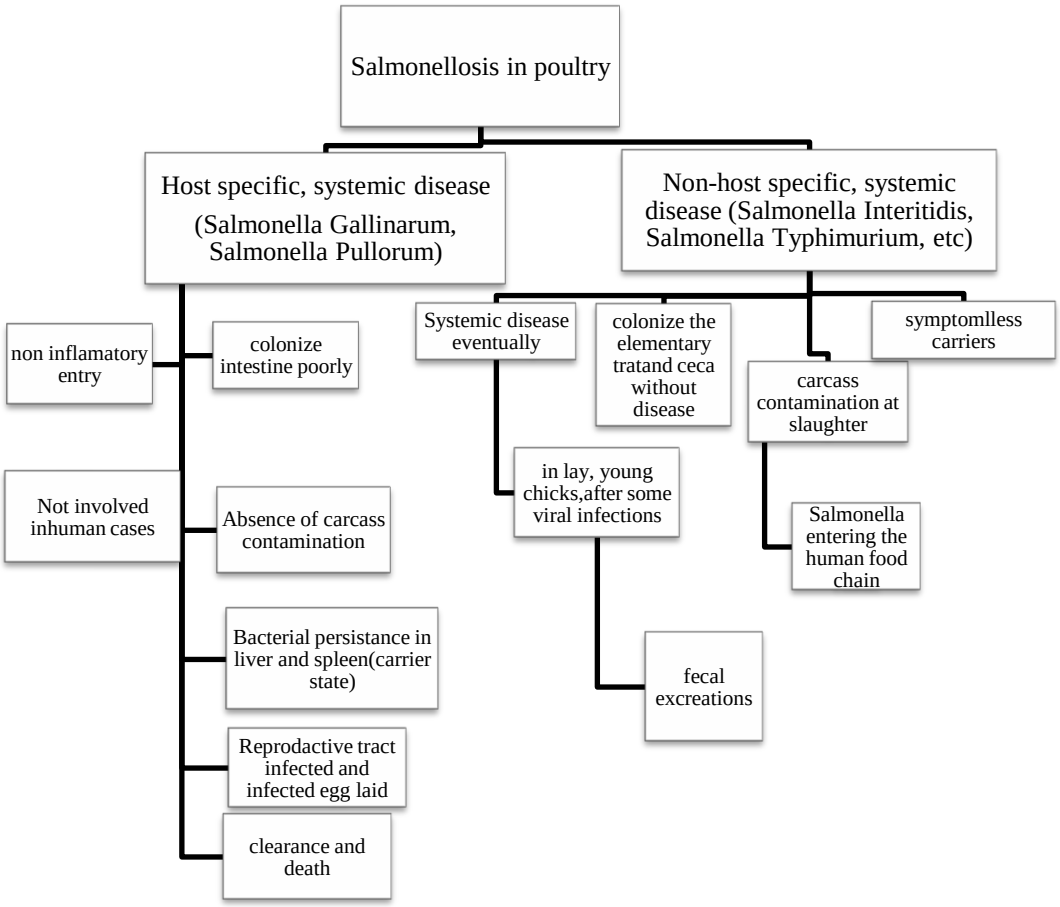


Figure 1- Biology of avian systemic salmonellosis.

2.4. Diseases in Chickens

Infection in chickens occurs at all ages and is characterized by severe hepatomegaly and splenomegaly accompanied by liver with bronzing aspect, anemia, and septicemia (Shivaprasad, 2000). The disease is dose-dependent and differences in pathogenicity may be found depending upon the susceptibility of the infected genetic line of chickens (Oliveira *et al*., 2005).

Birds infected with most *Salmonella* serovars do not show clinical signs of the disease, making it difficult to diagnose at the farm. However, S. Pullorum and S. Gallinarum do cause disease in poultry but rarely cause illness in humans. These *Salmonella* serovars are nonmotile and host-specific and cause Pullorum disease and fowl typhoid respectively (Rettger,1909). Pullorum disease was first described as "fatal septicemia" or "white diarrhea" (Rettger,1909). Clinical signs are predominantly observed in young chickens, showing lack of appetite, depression, respiratory distress, caseous core diarrhea, and early death a few days after hatching. In laying hens symptoms include reduced egg production, fertility, and hatchability (Lister and Barrow,2008). *Salmonella* Pullorum may cause severe systemic lesions including peritonitis and liver and spleen enlargement, and organs may be streaked with hemorrhages; animals can also develop white focal necrosis in the case of young birds and abnormal color and shape in ovaries in older birds.

Salmonella Gallinarum is primarily associated with the mononuclear phagocyte system and resides prematurely within macrophages in the liver and spleen. *Salmonella* Gallinarum can be found in the gastrointestinal tract early in the infection after oral contamination or at the final stage when the birds are dying (Barrow, 2007; Wigley *et al*., 2002). Regarding the epidemiology of fowl typhoid, the most important transmission route is horizontal, and very little information is available on direct evidences of transmission of the pathogen through the eggs to the progeny. *Salmonella* Gallinarum infection generally results either in mortality of susceptible birds or bacterial clearance in resistant birds within three to four weeks of the initial infection, although occasionally persistent infection may occur (Wigley *et al*., 2002; Wigley, 2004).

Mortality and morbidity rates due to fowl typhoid may reach up to 80%. Fowl typhoid has been eradicated from Australia, North America, and most European countries, where rigorous biosecurity and specific control programs including vaccination and good management practices have been largely applied. However, it is still of considerable economic importance in Ethiopia

and many countries of Africa, Asia, and Central and South America (Pomeroy & Nagajara, 1991; Lee *et al*., 2003). The most effective means of control is a combination of stringent biosecurity and management procedures and eradication (Calnek *et al*., 1997). Removal of birds that had died from disease from the environment and vaccinations regarded as a very useful measure for control of the fowl typhoid (Oliveira *et al*., 2005).

2.4.1. *Salmonella* Enteritidis Infection in Chickens

Chickens, excluding neonatal chicks, typically remain asymptomatic following the ingestion of *salmonella* Enteritidis. *Salmonella* Enteritidis excretion starts several hours after its ingestion and continues for several days. Chickens are highly resistant to *salmonella* Enteritidis infections during the rearing period. *Salmonella* Enteritidis excretion has been shown to continue for as long as several weeks. The susceptibility of laying chickens to *salmonella* Enteritidis infections varies with age; it is highest in neonatal chicks and most chicks die after its ingestion. Susceptibility then markedly decreases. *Salmonella* Enteritidis did not colonize the intestine in chickens that ingested 10^8 CFU/bird several days after birth, and no *salmonella* Enteritidis was isolated from the intestine (Burkholder *et al.*, 2008). However, susceptibility increases again at the laying age, with an increase being observed in the colonization rate after infection. It then slightly decreases. However, chickens at the laying age are less resistant to *salmonella* Enteritidis infection than those in the rearing period, facilitating colonization. Susceptibility increases as chickens grow. Susceptibility is increased by various stress factors (e.g., induction of molting) (Coward *et al.,* 2012). *Salmonella* Enteritidis has the highest affinity for eggs from *salmonella* bacteria. *Salmonella* Enteritidis is generally ingested orally by chickens, after which it colonizes the intestinal epithelium in the gastrointestinal tract. *Salmonella* Enteritidis has flagella and is in an active phase. However, *salmonella* Enteritidis ingested orally loses its flagella and is in a stationary (colonial) phase when it colonizes the intestinal epithelia. *Salmonella* Enteritidis expresses flagella again when it invades the solid organs through the intestinal tract wall in an active or stationary phase, and progresses to an invasive phase (Allen-Vercoe *et al*., 1997 and Bravo *et al.*, 2008). However, no study has been published on diarrhea in *salmonella* Enteritidis infected chickens. Thus, unlike *salmonella* Enteritidis infected humans and mice, *salmonella* Enteritidis in the intestinal epithelia of most *salmonella* Enteritidis infected chickens is presumably in a stationary phase and does not damage the intestinal epithelia of chickens. The

mechanism by which *salmonella* Enteritidis passes through the intestinal tract wall in laying chickens remains a matter of speculation. The mechanism underlying the phase transition from the active to stationary phase in *salmonella* Enteritidis in the intestinal tract of chickens has not yet been elucidated in detail. Macrophage cells have been shown to extend filopodia to *salmonella* Enteritidis in the intestines of *salmonella* Enteritidis infected mice. However, such a phenomenon has not been reported in chickens. Unlike other host-specific *salmonella* species which causes systemic disease after intestinal colonization in birds, *salmonella* Enteritidis belongs to the broad-host-range species and its infection in chickens is often silent, with the exception of very young chickens (three days), among which high mortality rates were observed. The asymptomatic status of *salmonella* Enteritidis infected chicken (also known as healthy carrier) has made it more difficult to control *salmonella* Enteritidis from spreading, therefore causes serious consequences on public health (Ohta and Toyota, 2012).

2.4.2. *Salmonella* Enteritidis Infection in Humans

In many countries *Salmonella enterica* serovar Enteritidis (SE) has become the first or second most common *salmonella* serotype (Barbour 1994). The epidemiology of *salmonella* Enteritidis accounts for the main sources of salmonellosis in human through the consumption of contaminated meat, eggs or other poultry products may trigger food poisoning, which is particularly dangerous for susceptible persons such as young children, elderly people and immuno-suppressed patients. Most people infected with *salmonella* develop diarrhea, fever, and abdominal cramps 12 to72 hours after infection. *Salmonella* Enteritidis can persist in the cecal or ovaries of adult birds for months without triggering clinical signs. The asymptomatic *salmonella* carrier status in poultry has serious consequences on public health. The colonized *salmonella* Enteritidis can be excreted in feces (horizontal transmission) or through the yolk (vertical transmission) to contaminate other birds in the flock as well as the poultry products such as meat (after slaughtering) and eggs (Barrow and Wallis, 2000).

Several *salmonella* species are pathogenic, some producing mild gastroenteritis, others producing a severe and often fatal food poisoning, which is called salmonellosis. Salmonellosis is caused by the infection with salmonella mainly through the route of ingestion. The common syndromes include diarrhea, fever, and abdominal cramps. Although most patients can recover from illness without antibiotic treatment in 4 to 7 days, the illness can be severe to elderly,

infants and those with impaired immune systems and cause death unless the person is treated promptly with hospitalization (CDC, 2013).

Salmonella Gallinarum and *Salmonella* Pullorum are highly host adapted, and they are not considered to be a serious public health concern. In one survey, only eight out of more than 450,000 isolations of *salmonella* from humans were *salmonella* Gallinarum. *Salmonella* Pullorum occasionally causes acute, self-limiting enteritis in people who eat massively contaminated food. The virulence of *salmonella* is related to its ability to invade host cells, replicate and resist both digestion and destruction against host defense system (OIE, 2012).

2.5. Prevalence and Distribution of Fowl Typhoid / Pullorum Disease in Ethiopia

Salmonella were considered to be one of the most economically important diseases of animals and human beings worldwide. Poultry has been the major reservoir of the infection. *Salmonella* Gallinarum which is the cause of fowl typhoid causes severe economic loss in poultry farms as the disease is characterized by high mortality and morbidity (Shivaprashad *et al*., 1997). In most of the developed countries such as Europe, North America and Australia the prevalence of fowl typhoid was almost nil as a result of serological testing and slaughter policies applied by them (Whiteman and Bickford, 1989).

Poultry production and consumption in Ethiopia, has been dramatically increased during last two decades and salmonellosis was reported as one of the prevalent diseases in commercial layers. Both S. Gallianrum as well as S. Pullorum is causing severe economical impact to the industry, while S. Enteritidis are also important in the public health aspects. Poultry has been the major reservoir of the Salmonellosis. There are epidemiological studies published on prevalence of Fowl Typhoid and Pullorum Disease in Ethiopia Abie *et al*. (2003) reported up to 97% mortality due to fowl typhoid which they confirmed by isolation in an outbreak in a farm in south western Ethiopia.

Genet *et al.* (2014) investigated the overall prevalence of *Salmonella* Gallinarum and *Salmonella* Pullorum in Haramaya University farm and Dire Dawa University farm were 31.8%. Kassaye *et al*. (2010), conducted a study to estimate the bacteriological prevalence of S. Gallinarum and Pullorum in apparently healthy chickens and to assess the proportional morbidity to mortality

from S. Gallinarum and Pullorum in sick or dead chickens in Hawassa from randomly selected chickens in 3 poultry farms. The prevalence of *Salmonella* infection (S. Gallinaum/Pullorum) was 0.8%. Aferaa *et al*. (2012) conducted a seroprevalence study of fowl typhoid using serum slide agglutination test was conducted in selected sites of East Shewa namely Debre zeit and Ziway District on a total of 814 chickens of commercial and local (backyard) types. The result shows that 44.6% recorded in backyard chickens and 26.6% in commercial poultry farms. Medina *et al.* (2013) conducted a cross sectional study with the objectives of determining the prevalence of poultry *Salmonellosis* by serology and media culture isolation from sero positive chicken of different eco-types kept under intensified production system 9.33% were positive to S. Gallinarum and/or S. Pullorum antibodies.

There are also some seroprevalence reports however, (Assefa, 1992, unpublished) reported a seroprevalence of 22.6% for chicken kept in small-scale farms in Addis Ababa, while seroprevalence of 10.4 - 28.3% has been reported for large scale commercial farms in Addis Ababa and Debre Zeit (Melese, 1991, unpublished). These preceding works have indicated that the diseases were highly prevalent and economically important diseases in chickens in different parts of the country (Berhea *et al.*, 2012; Dinka *et al.*, 2010 and Medina *et al*., 2013). The existence of the diseases especially in these local breeds of Ethiopia is of great concern as the diseases have the potential for horizontal and vertical transmission. The prevalence might have gone even higher if the sample size was increased and samples were taken from dead chickens.

The high prevalence of fowl typhoid may take the line share in the overall poultry production systems with high mortality rate and low productivity of backyard chickens in the country compared to the intensive production systems. In extensive production systems it was noted that there were history of occurrence of salmonellosis, minimal distance and lack of physical separation between different units, poor disposal of dead birds, absence of an all in all out systems, poor insecurity measures and maintenance of different types and multi age groups of birds in farm created favorable conditions for the outbreak and persistence of the disease (Dereje, 2002). These indicate that challenge of free ranging village poultry production and intensive poultry production may face in future the country from fowl typhoid and/or pullorum disease unless due attention is given to the prevention and control of these diseases.

2.6. The Interaction of *Salmonella* with the Host

Salmonella enterica are gram-negative facultative intracellular anaerobes that can invade a broad range of hosts causing both acute and chronic infections by means of their ability to replicate and persist within non-phagocytic epithelial cells as well as phagocytic dendritic cells and macrophages of the host innate immune system (Richter-Dahlfors *et al*., 1997, Yrlid *et al*., 2001). Epithelial cells and phagocytic cells such as dendritic cells, neutrophils, and macrophages identify specific pathogen-associated molecular pattern (PAMP) motifs and endogenous danger-associated molecular pattern molecules (DAMPs) present in the bacteria. Pattern-recognition receptors (PRRs), which include NOD-like receptors (NLRs) and TLRs, comprise the early components of the immune system that function to detect invading pathogens through PAMPs and DAMPs and signal to recruit and activate phagocytic cells such as neutrophils and macrophages (Kawai *et al*.,2010, Schroder *et al.*,2010). These receptors trigger an immune response and are key to establishing an important network between the innate and adaptive immune systems. Bacterial DNA, flagella, and LPS are examples of PAMPs, which activate TLR4, TLR5, and TLR9 signaling in the host. LPS-induced TLR4 activation is important for triggering the inflammatory responses of the host. It also plays an important role in mounting an inflammatory response to intravenously administered LPS. Mice with mutations in TLR4-encoding genes exhibit an increased susceptibility to *Salmonella* infection irrespective of other *Salmonella* resistance *loci* (Wilson *et al.,* 2008). Additionally, LPS plays an important role in the onset of sepsis during systemic infection as observed by its role in inducing inflammation in macrophages (O'Brien *et al.,* 2005).

The immune system can be divided into two main parts: the innate or non-specific and the adaptive or specific components. The innate immune system is the first host challenge presented to invading pathogens whereas the adaptive immune system provides further protection in addition to an immunological memory, which enables a faster response upon repeat exposure to the same pathogen or antigen. In addition to cellular components such as phagocytic cells, there are humoral elements such as the complement system that make up the innate immune system. Additionally, anatomical features like the mammalian skin layer act as physical barriers to infection. The interplay between the innate and adaptive immune systems, including different types of cells and molecules such as cytokines and antibodies, form the totality of the host

immunity. Leukocytes of the innate immune system include phagocytic cells, namely dendritic cells, macrophages and neutrophils, which can engulf foreign antigens, particles or pathogens. These phagocytic cells are recruited following the release of specific cytokine signals. These cells serve an important role in the activation of the adaptive immunity, which usually assumes the presence of lymphocytes (Janeway *et al.,* 2001). Other cells, such as basophils, eosinophils, and mast cells are also part of the host innate immune system that contributes to the innate immunity.

During the initial stages of an inflammatory response, neutrophils and macrophages are recruited to the site of infection. Neutrophils phagocytose the invading pathogens and kill them intracellularly. Similarly, macrophages and newly recruited monocytes, which will differentiate into macrophages following signaling or chemical stimulation, also function by phagocytosing and killing the pathogens at the intracellular level. Furthermore, macrophages are capable of killing infected or self-target cells and can also induce further downstream immune responses through the presentation of surface antigens to signal and recruit other cells and cell types (Delves *et al*., 2011).

A common feature of salmonellosis is the notable inflammatory response elicited by the host innate immune system. Both the host and pathogen have evolved defense mechanisms that result in a complex cross-talk that culminates with the induction of the host immune response. *Salmonella* species can cross the epithelial barrier by passive transport facilitated by dendritic cells, which extend pseudopods between local epithelial cells, or by active invasion. Upon reaching the lower intestine, the bacteria will adhere to the mucosal membranes and invade epithelial cells (Rescigno *et al*., 2001). One such site where this occurs is the microfold (M) cells of Peyer's patches that are located in the small intestine where the bacteria will translocate across the epithelial barrier to the underlying follicles and mesenteric lymph nodes of the lymphoid tissue (Jones *et al*.,1994**).** During sustained bacteremia, secondary infections can occur due to the dissemination of the bacteria to other organs such as the gall bladder, liver, and spleen. The gall bladder serves as a reservoir in chronic cases of *S*. Typhi and *S*. Typhimurium infection (Crawford *et al*., 2010, Gonzalez-Escobedo *et al.,* 2011). Infection by invading bacteria can originate from both the blood and/or retrograde bile. Biofilm formation on gallstones is a reported avenue through which chronic carriage and shedding of *Salmonella* species can be

established. These events set in motion a cycle of infection wherein bacteria basolaterally reinvade epithelial cells of the intestinal wall or are shed in feces. In time, the symptoms of salmonellosis will resolve. However, asymptomatic carriage of the bacteria can occur in patients for months or years with the potential to relapse in the future.

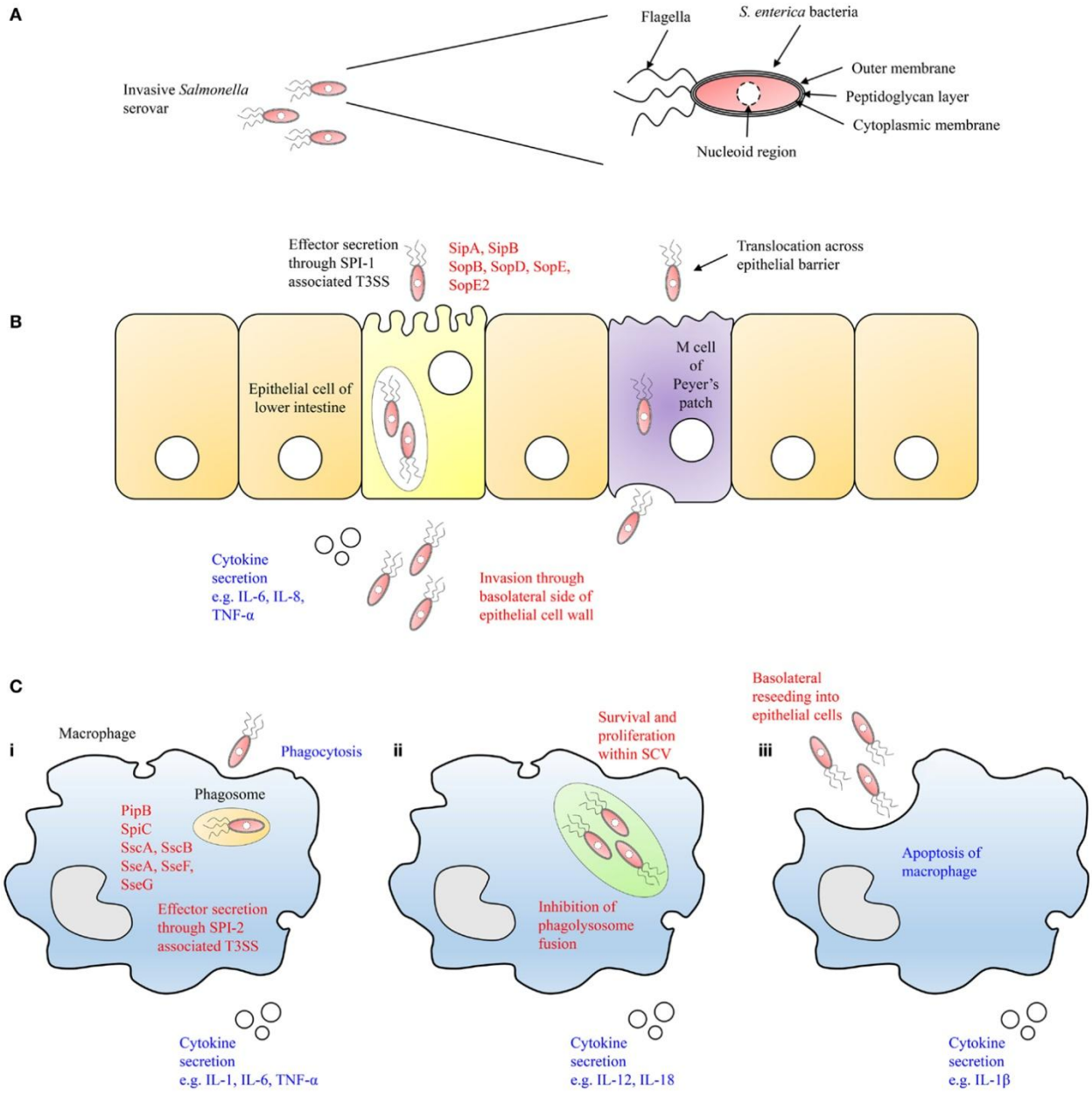


Figure 2. Schematic illustration of the infection of epithelial cells of the lower intestine and macrophages by *salmonella* is shown.

(A) The complex membrane structure of *salmonella* allows it to survive until reaching the epithelial cell wall of the host in the lower intestine. (B) *Salmonella* then translocate across M cells of Peyer's patches or actively invade epithelial cells by the secretion of effector proteins through the SPI-1 encoded T3SS-1. (C) (i) After crossing the epithelial barrier, *Salmonella* are engulfed by proximal macrophages that will secrete effector proteins into the cytosol of the cell via the SPI-2 encoded T3SS-2 and prevent fusion of the phagosome with the lysosome. (ii) Within the SCV, *Salmonella* will proliferate resulting in cytokine secretion by the macrophage. (iii) Finally, the macrophage will undergo apoptosis, and *Salmonella* will escape the cell to basolaterally reinvade epithelial cells or other phagocytic cells of the host innate immune system.

2.7. Immune mechanisms of protection: present and future perspectives

Despite the fact that many studies have been carried out to demonstrate the efficacy of inactivated (Woodward *et al*., 2002), sub-units , live attenuated (Atterbury *et al*., 2010; Kwon & Cho, 2011), bacterial ghost (Peng *et al*., 2011), genetically derivative strains (Nandre *et al*., 2011; Methner *et al*., 2011) used as vaccines, the efficacy of these product is demonstrated by challenge trials and recovery of the challenge strain. Little and isolated information explains how do vaccines work and the immune response depending on the antigen and route of vaccination. 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 & Ahlers, 2010). 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 & Powrie, 2008; Kelsall, 2008). An important difference must be established in *Salmonella* attenuated vaccines regarding the immune

response: 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; Mastroeni *et al*., 2000). 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 & 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 (Salazar-Gonzales & McSorley, 2005). In response to flagellin lamina propria dendritic cell, the differentiation of naive B cells into IgA plasma cells occurs via a mechanism that is independent of the gut associated lymphoid tissue (GALT) (Uematsu & Akira, 2009). Recognition of S. Typhimurium is largely mediated by TLR2, TLR4 and TLR5 (Uematsu *et al*., 2006; Vasquez-Torres *et al*., 1995) and TLR9 is involved in the regulation of the replication of the bacterium. Lypopolysaccharide (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 cells subsets are responsible for antigen presentation following mucosal vaccination (Belyakov & Ahlers, 2010). In the chicken, multiple lymphoid follicles exist, and they are made up of B cells embedded in a network of follicular dendritic cells (Brisbin *et al*., 2008). The chicken epithelial barrier can be

crossed using three mechanisms: endocytosis in the intestinal epithelial cells, transcytosis crossing M cells (microfold cells), and directly through the intraepithelial lymphocytes.

The third step in the immune response produced by oral vaccines is the processing by the dendritic cells and the presentation to the T cells. Dendritic cells in the gut can be activated by epithelial cells, which produce cytokines based on the invasiveness of the bacteria, and directly by noninvasive bacteria (Rimoldi *et al*., 2004). *Salmonella* vaccines administered orally must induce Th1 and Th2 (T helper cell subset 2) responses, stimulating cell-mediated immunity and B cell activation for producing SIgA, which block the attachment of the bacteria to mucosal surfaces. 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 *et al*., 2005), 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). Mucosal memory T cells phenotypes differ substantially depending on the regimen of immunization, a secondary responses result in preferential accumulation of memory T cells in the lamina propria following mucosal vaccination (Barnhart *et al*., 1991). These cells might be exploited to develop new live attenuated vaccines inducing abroad repertoire of immune responses against intracellular pathogens (Titball, 2008).

Vaccination is an excellent tool for handling poultry *salmonella* prevention programs in developed and developing countries. Vaccines against *salmonella* infections in chickens and other food-producing animals require protection at both mucosal (gut) and systemic levels. There is no ideal product, but regarding the immune response, live attenuated *salmonella* vaccines are superior to inactivated, deleted or subunit vaccines. Oral *salmonella* vaccines provide an advantage over parenteral products, because oral products are able to mimic natural infection and to stimulate both the mucosal and systemic immune responses, whereas parenteral products are able to generate only systemic immune responses (Lilina, 2012).

2.8. Vaccination

Vaccination is an inexpensive and effective method in the control of pathogenic organism. The concept of vaccination is to challenge the host immune system to provide antibodies (immunoglobins) to the challenge. These vaccines have been delivered to poultry in many modes, including in *ovo*, intramuscular, spray, eye drops, and drinking water (Sharma, 1999). Administration results in the immune system developing a memory, in the form of specific T and B memory lymphocytes, to the challenge and subsequent wild challenges are eliminated prior to establishment (Goldsby *et al.*, 2003).

Vaccination against viral (Newcastle and Mareks disease) and bacterial (*Mycoplasma*, *Pasteurella*) pathogens of veterinary health concern, including some *salmonellae* (such as serotypes Pullorum and Gallinarum), is a widespread and longstanding practice (Nair, 2005). Vaccination for eradication of systematic infection by the host-specific serotypes Pullorum and Gallinarum, as in an early report indicated (Smith, 1956), is still effective today.

The use of vaccination against pathogens of public health concern is a much more recent development and has been limited largely to serotypes of prevalence (e.g. Enteritidis and Typhimurium). Both live (e.g. *aroA* deletion) and killed vaccines (Barrow, 2007) have been developed, with some studies (Bailey *et al.*, 2007a) using a combination vaccine. This combination vaccine program started with an attenuated then killed *salmonella* serotypes thus stimulating both cell mediated and humoral immune systems, resulting in higher titres than individual vaccination programs (Bailey *et al.*, 2007a). Vaccination to prevent or reduce *Salmonella* infection in poultry has been accepted worldwide. Although both live and inactivated vaccines have been prepared for use against *S.* Gallinarum (Paiva *et al.*, 2009), the vaccine most widely used is made from the rough 9R strain (Harbourne *et al.*, 1963). It is normally only employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination. Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection.

Autogenous or locally produced vaccines can also be used to control clinical disease, but care must be taken to avoid strain instability leading to reversion to virulence (Okamoto *et al.*, 2010). Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks. More recently, vaccines derived from outer membrane proteins, mutant strains, and a virulence-plasmid-cured derivative of S. Gallinarum have shown promise in protecting birds against challenge.

2.8.1. Current vaccine in Ethiopia

Salmonella Gallinarum causes fowl typhoid in chickens and has been of economic importance to the chicken industry in many countries. The biovar Gallinarum live vaccine strain 9R (SG 9R) has been used to control fowl typhoid in many areas where the disease is endemic. Presently, live attenuated *salmonella* Gallinarum 9R vaccine (in freeze-dried form) was produced in Ethiopia (National Veterinary Institute). Each dose of reconstituted vaccine contained not less than 20 million viable organisms (OIE, 2012). The vaccine was administered by subcutaneous injection in to the back of the neck. The dosage volume was 0.5 ml and the age for priming vaccination was 8 weeks, with re-vaccination at 18 weeks of age. Commercial layer and broiler industries in Ethiopia have gradually accepted routine vaccination as a preventive intervention method to reduce the *salmonella* in industrial farms.

2.8.2. Vaccine Efficacy test

Vaccination may play the vital role for effective prevention and control of fowl typhoid. Vaccination may reduce flock losses, but will not prevent infection with field strains (Silva *et al*., 1981). The 9R strain vaccine had already been developed in the 1950s but there were not many reports about the efficacy and the safety of the strain. Feberwee *et al*. (2001a,b) reported an evaluation of efficacy, safety and serologic responses in layers more than 6 weeks old with the 9R vaccine in The Netherlands. However, the purpose of the test was only to determine protection against *Salmonella* Enteritidis. The 9R strain has a semi-rough lipopolysaccharide structure that reduces the virulence of that microorganism (Feberwee *et al*., 2001a,b). This vaccine may also provide some protection against *Salmonella* Enteritidis and *Salmonella* Typhimurium (Barrow *et al*., 1992; Audisio & Terzolo, 2002). *Salmonella* Gallinarum 9R

vaccine presented acceptable safety and efficacy in young layer hens even when administered at 4 weeks of age (Lee *et al*., 2005). In addition, no evidence of fecal shedding of the vaccine strain was found (Feberwee *et al*., 2001). However, there have been reports of SG9R vaccine strain fecal shedding for a maximum time of 24 hours after vaccination. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at postmortem exceeds by eight or more the number of similarly defined control chickens (OIE, 2012).

3. MATERIALS AND METHODS

3.1. Study Area

The present study was conducted from November, 2014 - June, 2015. Sampling areas were selected for the study based on occurrence of salmonellosis outbreaks affecting the major areas of central Ethiopia where poultry farming activities are highest. During this period a total of 14 salmonellosis outbreaks were recorded from these areas (Table 2). All the farms practiced the deep litter management system. Bacterial isolation and characterization and the challenge experiments were under taken at the National Veterinary Institute, Ethiopia, located in Bishoftu town.

Table:2: Sample collection areas from different laying poultry farms suspected of salmonellosis and number of animals euthanized.

S/N	Sample collection area	No of farms	No of eutanized chicken
1	Addis Ababa	4	4
2	Adama	1	1
3	Awash Malkasa	1	1
4	Bishoftu	6	9
5	Dukem	1	1
6	Zaway	1	1
	Total	14	17

3.2. Specimen collection

Salmonella strains used in the current study were obtained from specimens collected from suspected cases from 14 laying poultry farms suffering from loss of production and sudden death. Specimen from tissue with typical pathological lesions found in different organs (liver, heart, spleen, ovary and caecal junction) were collected aseptically after euthanizing clinically suspected chicken. All the samples were obtained from all ages. In all the farms, the chicken were not vaccinated against salmonellosis.

3.3. Isolation and identification of *salmonella* isolates

3.3.1. Specimen processing and isolation

The samples were homogenized and pre-enriched in buffered peptone water for 24 h at 37 ◦C, followed by an enrichment step in Rappaport-Vassiliadis broth for 48 h at 42◦C. Droplets of the Rappaport-Vassiliadis broth were spread onto Brilliant Green Agar and SS agar and incubated for 48 h at 37◦C. Also materials from samples were also directly Gram stained to detect any bacteria in the sample before cultivation. Colonies showing the cultural and morphological characteristics of *salmonella* was sub-cultured to get pure cultures for further biochemical and molecular identification and characterization.

3.3.2. Biochemical characterization

Biochemical test for *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Enteritidis including indole test, methyl red, voges-proskauer reaction, citrate utilization, nitrate reduction, urea hydrolysis, triple sugar iron agar, dulcitol and ornithine were done. And a motility test media was used to confirm non-motility. Biochemical tests were used to distinguish biotype Gallinarum from biotype Pullorum. Specifically, biotype Gallinarum strains, in contrast to biotype Pullorum strains, are able to ferment maltose and dulcitol and unable to ferment rhamnose, and are ornithine decarboxylase positive. *Salmonella* Enteritidis differ from the other two biotypes because of its motility.

3.3.3. Molecular identification

DNA Extraction

DNA extraction was done using DNeasy Blood & Tissue kit (Qiagen, Germany) according to manufacturer's instructions. An overnight pure culture of the bacterial isolate at log phase of growth for each isolate was used for DNA extraction.

Multiplex Polymerase Chain Reaction(mPCR)

Molecular identification of the isolates were carried out using primers flanking genes specific for each serotype in by multiplex PCR described by Yang *et al*., (2014) with some modifications. The list of primers used for identification of the serotype are listed in table 3. The amplification reaction was carried out in a final volume of 27µl which are 2µl Rnase free water, 2µl of 2pmol/µl each primer pair, 10µl iQ ^{TM} Super mix (BIO-RAD, USA) and 3µl each templates were added (ISO IEC 17025:2005 NVI QMS SOP). Finally the mix was incubated in a thermal cycler (Applied Bio-systems) with: initial denaturation at 94 ^{0}C for 5 minutes, denaturation at 94 ^{0}C for 60 seconds, annealing at 55 ^{0}C for 60 seconds and extension at 72 ^{0}C for 60 seconds and run for 35 cycles and follow with 5 minutes at 72 ^{0}C for final elongation step and hold at 4^{0}C until analysis. 3µl of each PCR product was loaded on a 2% agarose gel stained with Etidium bromide solution and a parallel lane was also be loaded with a 1Kbp DNA-marker ladder (Fermantas) in TAE Buffer (Tris-acetate-EDTA). The products were separated on agarose gel electrophoresis system (BIO-RAD, USA) at 120 V for 80 minutes, the gel was documented and photographed under ultra violet light (Uvitec, UK). The size of the PCR product flanked by the primers are 174bp and 252bp for *Salmonella* Gallinarum, 252bp for *Salmonella* Pulorum and 304bp for *Salmonella* Enteritidis.

Table 3: *Salmonella* specific primers used in characterization of *salmonella* serotypes.

Specific genes	Name of primers	Primer sequence	Product size (bp)
Sdf I	SE-F	TGT GTT TTA TCT GAT GCA AGA GG	304
	SE-R	TGA ACT ACG TTC GTT CTT CTG G	
SlgC	SGP-F	CGG TGT ACT GCC CGC TAT	252
	SGP-R	CTG GGC ATT GAC GCA AA	
Spec	SG-F	GAT CTG CTG CCA GCT CAA	174
	SG-R	GCG CCC TTT TCA AAA CAT A	

SG = S. Gallinarum, SGP = S. Gallinarum-Pullurom, SE = S. Entratidis

3.4. *Salmonella* Gallinarum 9R vaccine

The live attenuated *Salmonella* Gallinarum 9R vaccine (National veterinary Institute, Ethiopia) batch number of Ft 1/2015 was used in this study. *Salmonella* Gallinarum 9R is a live, freeze-dried vaccine, produced based on the rough *Salmonella* Gallinarum strain 9R which contains $2x10^7$ CFU per dose.

3.5. Experimental challenge and vaccine evaluation study

The challenge experiment was under taken at the animal challenge facility of the National Veterinary Institute located in Bishoftu town from November, 2014 - May, 2015.

3.5.1. Experimental animals and management

A total of 90 chicken of white leg horn breed obtained from National Veterinary Institute which were reared for SPF egg production, were used for the experimental challenge and vaccine efficacy trial. The chicken were reared and fed according to the recommendations of the animal production manuals and protocol developed at National Veterinary Institute animal handling facility. At the age of 6 weeks birds were examined according to Zancan *et al*., (2000) to confirm whether they were free from *salmonella* infection and for any antibodies against *salmonella* using rapid serum plate agglutination test. The birds which gave negative reaction to *salmonella* antigen were used for the experiment. Throughout the experiment, chicken were provided separately with water and antibiotic free feed. All animal handling and experiments were done according to the animal handling and use guidelines stipulated by Research and Ethics Committee of Addis Ababa University, College of veterinary medicine and Agriculture.

3.5.2. Experimental design, immunization and challenge experiment

The birds were randomly categorized into two groups for the purpose of vaccination each comprising 45 birds. The first group was vaccinated with live attenuated *salmonella* Gallinarum 9R vaccine while the second group was kept as non vaccinated control. Each of these categories were further divided and randomly assigned into 3 replicate groups each comprising 15 birds. Each group was labeled and kept in separate cages. The replicate groups (both vaccinated and

unvaccinated) were challenged with S. Gallinarum, S. Pullorum and S. Entratidis isolated from cases of salmonellosis.

Immunization

At the age of six weeks the first group chickens were immunized with 0.2 ml (one dose) of the reconstituted vaccine per bird by subcutaneous injection into the lower part of the back of the neck, and the remaining 45 birds left for control. The live attenuated *Salmonella* Gallinarum 9R vaccine was used in this study. *Salmonella* Gallinarum 9R is a live, freeze-dried vaccine, produced based on the rough *Salmonella* Gallinarum strain 9R which contains $2x10^7$ CFU per dose.

Challenge experiment

The Challenge strains were S. Gallinarum, S. Pullorum and S. Entratidis isolated from a case of salmonellosis. Broth culture was made by subculturing from the frozen stock culture. Inoculums of each isolate of the culture was prepared by using sterile saline. The density of the inoculums were adjusted to 0.5 McFarland standard (HIMEDIA) which was estimated to be comparable to a bacterial cells of $5x10^7$ CFU/ml and based on plate count agar (Lee *et al.,* 2005).

The challenge was conducted two weeks post vaccination. Both vaccinated and unvaccinated chickens of each group were deprived of food for approximately 6 hours followed by oral administration of 1 ml of bacterial suspension containing 5×10^7organisms of each of the isolates.

3.5.3. Post immunization and post challenge follow-up

The immunological status of the vaccinated and unvaccinated groups was monitored by routine serology. Blood samples were collected from each hen at the time of vaccination, 1st and 2nd week of post vaccination. Rapid Serum slide agglutination test was performed according to Quinn (1994). Samples were assayed for the presence of specific antibodies by using RSAT. Challenged birds were observed for 2 weeks during which development of clinical signs and daily mortality was recorded. Post mortem examination were conducted in all birds that died during the challenge period. Liver and spleen samples were taken for isolation of salmonella strains employing the procedures described above. For bacterial recovery, a sample of the liver

and spleen was minced in buffered peptone water and incubated overnight at 37^0C with continuous shaking, followed by culture in Rappaport- Vassiliadis broth for 48 h at 42^0C. A loop of the enrichment broth was streaked onto BGA and the salmonella- type colonies were examined after incubation at 37^0C for 24 h.

3.6. Statistical analysis

Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All results are expressed as means ± standard error of the mean (SEM), unless otherwise specified. One way ANOVA was used for analysis of protective efficacy and lesion scoring. The chi-square test was performed for significant differences of mortality post-challenge between the immunized and control groups. Statistical significance was identified as p-value < 0.05.

4. RESULTS

4.1. Isolation and identification of S. Gallinarum, S. pullorum and S. Entritids

A study on the isolation of salmonella species from specimens collected from cases of chicken resulted in bacteria 14 isolates that grew on SS agar with whitish or slight grayish colonies and dark central spot indicative of hydrogen sulfide production. Culturing the same isolates on TSI agar showed alkaline slant (red color). In Gram's staining, the bacterial isolates were gram-negative small rods occurring as single or paired. All the isolates fermented glucose and mannitol but did not ferment lactose and sucrose. Some *salmonellae* (92.86%) fermented maltose and some (7.14%) did not. All of the isolates were MR positive but VP and indole were negative.

The cultural, colony and biochemical features of the isolates were found to be consistent with characteristics of *salmonella* species. In motility test, 13 of the isolates were non motile while one was motile indicating the former to be either S. Pullorum or S. Gallinarum and one of the isolate to belong to other *salmonella* species. Twelve of the 13 non-motile *salmonella* isolates fermented dulcitol showing their presumptive identification as *Salmonella* Gallinarum**.** One nonmotile isolate did not ferment dulcitol. When one isolated organism produces acid and gas in dextrose, mannitol and xylose but did not ferment maltose and dulcitol, it is considered that the isolate is S. pullorum (OIE, 1996). The dulcitol fermentation test is performed to differentiate non-motile *S.* Pullorum and *S.* Gallinarum worldwide (Robinson *et al*., 2000; Rahman, 2003). Based on additional biochemical tests, the 14 isolates obtained in this study were finally presumptively identified as one isolate of *Salmonella* Pullorum (SP), 12 *Salmonella* Gallinarum (SG) and 1 motile *salmonella* (Table 4).

Table 4- Biochemical reactions used to differentiate between *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Enteritidis.

Reactant or property	Isolate A	Isolate B	Isolate C
Dextrose	Fermented with no gas	Fermented with gas	Fermented with no gas
Lactose	Not fermented	Not fermented	Not fermented
Sucrose	Not fermented	Not fermented	Not fermented
Mannitol	Fermented with no gas	Fermented with gas	Fermented with no gas
Maltose	Fermented with no gas	Usually not fermented	Fermented with no gas
Dulcltol	Fermented with no gas	Not fermented	Fermented with no gas
Ornithine	Not fermented	Fermented	Not fermented
Rhamnose	Fermented	Fermented	Fermented
Indole	Not produced	Not produced	Not produced
Urea	Not hydrolysed	Not hydrolysed	Not hydrolysed
Motility	Non-motile	Non-motile	Motile
Interpretation:	S.Gallinarum	S.Pullorum	S.Enteritidis

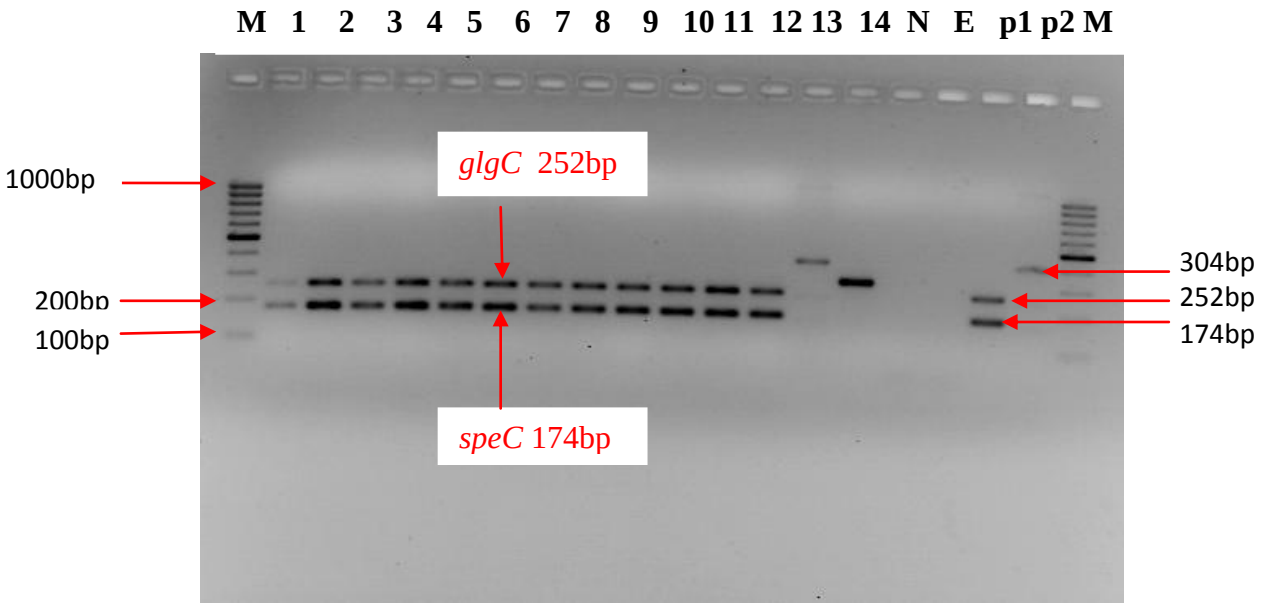
Table 5- Total number of isolated bacteria from euthanized chickens suspected of salmonellosis.

Total no of outbreaks suspected of Salmonellosis	S. Gallinarum	S. Pullorum	S. Enteritidis
14	12(85.7%)	1(7.1%)	1(7.1%)

4.2. Results of multiplex PCR

Multiplex PCR run with three pair of primers for characterization of the *salmonella* isolates from poultry, the 174bp and 252bp bands produced by *speC* gene and *glgC* gene respectively (specific for S. Gallinarum), 252bp band produced by *glgC* gene (specific for S. Pullorum) and 304bp produced by *sdf I* gene (specific for S. Enteritidis) (figure 3). Twelve samples were amplified *speC* and *glgC* genes as shown by double bands indicates *Salmonella* Gallinarum, one

Salmonella Enteritidis was amplified at 304bp and *glgC* gene amplified at 252bp indicates *Salmonella* Pullorum. The molecular identification employing multiplex PCR with species specific primers, the results were consistent with the phenotypic (presumptive) identification which further confirmed the isolates to be *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Enteritdis.



M- Molecular marker (Fermentas)

Sample No 1- 12 were positive around 174bp and 252bp.

Sample No 13 was positive around 304bp.

Sample No 14 was positive around 252bp.

N- Negative control without template - no amplification

E- Extraction control (Rnase free water) - no amplification

P1 and P2 - Positive controls (SG vaccine Seed, SE local isolate)

Figure -3. Agar gel electrophoresis of mPCR of 14 *salmonella* samples obtained from cases in some poultry farms.

4.3. Immune response of the *salmonella* Gallinarum vaccine

Assessment of immune status against *salmonella* infection in chicken used for the challenge experiment just before inoculation of *Salmonella* Gallinarum 9R vaccine showed all were sero-negative. The results of the immune response of vaccinated birds is presented in table 6. The percentages of chicken detected as sero-positive after vaccination ranged from 80% at 1st week post vaccination to peak values of 95.6% by the 2nd weeks post vaccination.

Table 6- Antibody response of birds after vaccination with *Salmonella* Gallinarum 9R vaccine.

Time intervals	No. of tested Samples	No. of positive samples	%
0	45	0	0
7 days	45	36	80
14 days	45	43	95.6

4.4. Protection against challenge

The protective and cross protective efficacy of the live *Salmonella* Gallinarum 9R vaccine is presented in Table 7. In the vaccinated group, no mortality was observed in *Salmonella* Gallinarum challenged group despite that three chicken showed slight depression and weakness. Pinpoint hemorrhages on the liver and enlarged spleen during post-mortem examination were observed in these chicken. However, in the non-vaccinated group, 8 (53.3%) birds died, 5 (33.4%) birds showed macroscopic lesions and the remaining 2 (13.4%) showed no lesion.

The mortality and lesion scores after challenge with the *Salmonella* Pullorum virulent strain, one chick in the immunized group showed macroscopic lesion, while in the un-immunized group 10 (66.7%) chicken showed prolonged depression and 13 (86.7%) chicken showed post-mortem lesion. No mortality was observed in the immunized group while 2 of 15 birds died in the un-immunized group.

The mortality and lesion scores after challenge with the *Salmonella* Enteritidis virulent strain showed that no macroscopic lesion was observed in the immunized group. Slight depression was

observed in 9 (60%) of the un-immunized chicken and 10 (66.7%) showed postmortem lesion. No mortality was observed in both immunized group and un-immunized group. The mortality rates and lesion scores in vaccinated and unvaccinated groups is presented in Table 7.

Table 7- The mortality and lesion scores after challenge with the S. Gallinarum, S. Pullorum and S. Enteritides virulent strain.

	Protection against SG		**Cross protection against SP**		**Cross protection against SE**	
	Vaccinated	Control	vaccinated	Contol	Vaccinated	Control
No of chicken	15	15	15	15	15	15
Mortality(%)	0(0%)	8(53.4)	0(0%)	2(13.3)	0(0%)	0(0%)
Lesion score(%)	3(20%)	5(33.3%)	1(6.7%)	13(86.7%)	0(0%)	10(66.7%)

Control represent unvaccinated group

Table 8- Comparison of the three challenge isolates virulence and pathogencity using one-way ANOVA analysis.

No. of hen	**Challenge isolate**	**Mortality**				**Lesion development**			
		proportion	95% CI	F	p-value	proportion	95% CI	F	p-value
15	SG	0.53 (8)	0.25 - 0.82	8.878	0.001*	0.87 (13)	0.67 - 1	1.235	0.301
15	SP	0.13 (2)	0.00 - 0.33			0.87 (13)	0.67 - 1		
15	SE	0	-			66.7 (10)	0.40 - 0.94		

* = P < 0.05 considered statistically significant

SG = S. Gallinarum, SP = S. Pullurum, SE = S. Entratidis

Table 9 - Comparison of protective efficacy of S. Gallinarum 9R vaccine against the three challenge isolates

Challenge type	Group	No. of birds	No. of birds having lesion	Protection (%)	Vaccine efficacy (%)
S. Gallinarum	Vaccinated	15	3	80.00%	76.9%
	Unvaccinated	15	13		
S. Pullurum	Vaccinated	15	1	93.30%	92.3
	Unvaccinated	15	13		
S. Entratidis	Vaccinated	15	0	100%	100%
	Unvaccinated	15	10		

The current study showed that no significant difference between *Salmonella* Gallinarum and *Salmonella* Pullorum in terms of virulence and the live vaccine *Salmonella* Gallinarum 9R strain provided significant ($P < 0.05$) as well as cross protection against challenge with S.Gallinarum, *Salmonella* Pullorum and *Salmonella* Enteritidis.

4.5. Bacterial Recovery after challenge

The bacteria strains used for challenge (S.Gallinarum, S.Pullorum and S.Enteritidis) from the respective group were re-isolated from internal organs including the liver, spleen, and caecum of 5 randomly selected birds from each group at 14 days after challenge. Colonies recovered on BGA were found to have typical cultural and biochemical features of the inoculated *Salmonella* strains.

Table 10 - Recovery of challenge strains from the internal organs of chicken.

challenge strains	S.Gallinarum		S.Pullorum		S.Enteritidis	
	Vaccinated	control	Vaccinated	Control	Vaccinated	Control
No of samples	5	5	5	5	5	5
Bacterial Recovery	0(0%)	3(60%)	0(0%)	4(80%)	0(0%)	4(80%)

5. DISCUSSION

The colonies of bacteria isolates in SS agar, TSI agar and BGA had features that conform to *salmonella* species as reported previously (Perez *et al.,* 2004). The results of grams reaction, cell morphology and biochemical characteristics were in agreement with the findings of previous similar studies which were consistent with the characteristics of *salmonella* (Sujatha *et al.,* 2003; Khan *et al.,* 1998). Although the results of motility tests and the fermentation of dulcitol enabled presumptive identification the isolates as S.Pullorum and S.Gallinarum (OIE, 2004) it was not possible to identify the serotypes of one of the *salmonella* isolate. Further characterization of the isolates based on molecular method using multiplex PCR (mPCR) assay was not only confirmed the serotypes identified phenotypically but also further discriminated the unknown serotypes as S.Enteritidis. This showed the fidelity and discriminatory power of the multiplex PCR to provide rapid and definitive detection of avian *salmonella* serotypes (Shah *et al.,* 2005).

During the last decade, a number of studies have demonstrated the practicality of identifying *salmonella* serovars using multiplex PCR (mPCR) (Kim *et al.,* 2007). In addition, the technique has been shown to be a powerful and cost-effective tool for *salmonella* serotyping (Ben *et al.,* 2010). The method is based on detection of *glgC* and *speC* genes which are present in specific serotypes and are able to discriminate between biovars Gallinarum and Pullorum. While the primers flanking the *speC* and *glgC* genes used yield amplicon from biovar Gallinarum the *speC* gene does not yield any amplicon from biovar Pullorum (Kang *et al*., 2011).

Developing protection measures of chickens against *salmonella* infections, including fowl typhoid, is one of the most important issues in the poultry industry (Lee *et al.,* 2005; Van Immerseel *et al.,*2005). Although both vertical and horizontal transmissions have been suggested to occur, *Salmonella* Enteritidis has been disseminated worldwide by poultry through vertical transmission (Wall & Ward, 1999). *Salmonella* Gallinarum is an intracellular pathogen that invades the gastrointestinal mucosa and establishes systemic infection after being carried in macrophages (Chappel *et al.,* 2009). Thus, development of an efficient systemic immune response requiring both cell-mediated and humoral immune responses is supposed to be essential for protection (Wigley *et al.,* 2002).

A number of workers (Smith *et al*., 1956; Harbourne *et al.,* 1957) have demonstrated the value of rough 9R vaccine as a means of prevention against fowl typhoid. Harbourne *et al*., 1963 confirmed the efficacy of the 9R strain by conducting a field trial with fowl typhoid-infected flocks. Live vaccines produce better protection against *salmonella* than killed ones (Gast *et al.*, 1993). It has been demonstrated that killed vaccines can induce effective protection against invasion of visceral organs but fail to control intestinal colonization by *salmonella* (Gast *et al*., 1993; Nakamura *et al*.,1994).

The immune response due to vaccination using *salmonella* Gallinarum 9R in a significant proportion of birds complements the findings of Yamane *et al.,* (2000) and Barrow *et al.* (1990). The current study concedes also with the findings of Feberwee *et al.,* (2001a) which showed that *salmonella* Gallinarum 9R vaccine provided significant protection against challenge with *salmonella* Gallinarum as well as cross protection against challenge with *salmonella* Enteritidis. This vaccine may also provide some protection against *salmonella* Typhimurium (Barrow *et al*., 1990; Audisio & Terzolo, 2002).

The cross protection by *Salmonella* Gallinarum, 9R strain vaccine observed in the current study is due to the fact that *Salmonella* Gallinarum*, Salmonella* Pullorum and *Salmonella* Enteritidis are classified as group D *Salmonella* (Kauffman- White scheme) implying that they share similar antigenic structure (O-antigens 1, 9 and 12) (Ochoa-Repa´raz *et al.,* 2004; Forshell & Wierup, 2006).The higher virulence of the wild strain of S. Gallinarum as observed in susceptible non vaccinated chicken might be associated with its immunogenicity. The high level of antibody response in vaccinated hens was considered to be due to high agglutination property of the bacterial cells (Wigley *et al*., 2005).

The absence of *salmonella* strains during re-isolation from internal organs in the vaccinated chicken unlike that of unvaccinated group challenged with any of the *salmonella* strains indicates effective clearance of the bacteria by the immune system in vaccinated groups by the time of sampling.

6. CONCLUSIONS AND RECOMENDATIONS

The virulent challenges were performed with a wild-type *Salmonella* Gallinarum, *Salmonella* Pullorum and *Salmonella* Enteritidis strains isolated from outbreaks of poultry salmonellosis. All chickens of the unvaccinated groups showed detectable changes in their general condition. By contrast, the vaccinated chickens showed no or much reduced clinical signs. The death rates of the vaccinated groups were significantly ($P < 0.05$) reduced compared with the corresponding control groups. Examination for macroscopic lesions and the absence of the challenge strains in internal organs such as the liver and spleen indicates effective clearance of the bacteria by the immune system in vaccinated chickens.

Therefore vaccination with live *Salmonella* Gallinarum 9R strain can provide effective protection against natural infections of *Salmonella* Gallinarum as well as cross-protection against *Salmonella* Pullorum and *Salmonella* Enteritidis providing its usefulness as a vaccine against fowl typhoid.

Based on these findings the following recommendations are suggested:

- ✓ The protective efficacy of the vaccine should be monitored routinely against outbreaks.
- ✓ Currently available vaccine have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Therefore further studies should be conducted on the vaccine to generate long-lasting protection.
- ✓ Frequency of Mutations in *Salmonella* is relatively high, especially undefined mutations, the vaccine should be shown to be stable and checks on stability should be made by molecular fingerprinting methods or sequencing.

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8. LIST OF APPENDICES

Appendices 1-Culture Media preparation

MacConkey agar (CONDA)

Suspend 50g in 1liter distilled water, mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. completely. Sterilize in autoclaving at 121^0c for 15minutes.Cool to 45- 50^0C mix well and dispense into plates. Allow the plates to solidify and place them upside down to avoid excessive moisture on the surface of the medium.

Tryptose broth (Oxoid)

Dissolve 26g in 1000ml distilled water stir and dissolve completely. Sterilize by autoclaving at 121^0c for 15 minutes.

Peptone water (HIMEDIA)

Dissolve 15g in 1000ml distilled water mix well and dispense in to tubes. Sterilize by autoclaving at 15lbs pressure (121^0c) for 15 minutes.

Oxidation-Fermentation test media

Suspend the dry ingredients in 1liter distilled water. Heat to boiling, to dissolve completely. Autoclave for 15 minutes at 121^0c. After cooling the medium to 55^0c, aseptically add the carbohydrate solution of choice and mix completely. Note that the carbohydrate solution should be sterilized by filtration through a 0.2µm membrane filter; heat sterilization may decompose some carbohydrates. Dispense 5ml aliquots into screw cap test tube, and allow the medium to solidify. The medium should be P^H 7.1 at room temperature. Test for oxidative utilization are performed by inoculating the medium and incubating for 24 - 48 hours. Tests for fermentative utilization are performed as above, but the tubes are over laid with 1cm of vaspar (a mixture of Vaseline and paraffin).

Selenite Broth (Selenite F broth)(TITAN Biotech)

Dissolve 23g in1000ml distilled water. Stir and dissolve completely. Gently heat, avoid excess heating. Do not autoclave. Sodium selenite is toxic and potential tetraogen.

Nutrient agar (Oxoid)

Suspend 21g in 1liter cold distilled water. Heat to boil, to dissolve completely. Dispense into appropriate containers and autoclave for 15minutes at 121^0c. The medium should be P^H 6.8 at room temperature. Cool to45^0c and dispense into sterile petridishes.

***Nutrient* broth (*TITAN Biotech*)**

Suspend 8g in 1liter cold distilled water. Heat to boil, to dissolve completely. Dispense into appropriate containers and autoclave for 15minutes at 121^0c. The medium should be PH 6.8at room temperature. Cool to 45^0c and dispense into sterile petridishes.

Lysine Decarboxylase broth (CDH)

Suspend 14g in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Dispense 5ml in to screw caped test tubes. Sterilize by autoclaving at 15lbs pressure (121^0C) for 15 minutes. Allow the tubes to cool in upright position.

Brilliant Green Agar (Fluka)

Suspend 52g in 1liter distilled water. Heat gently with occasional agitation and bring just to dissolve the medium completely. Do not autoclave Cool to 50^0C ,mix well and pour plates.

Salmonella Shigella Agar (TITAN BIOTEACH)

Suspend 63g in 1000ml distilled water. Boil with frequent agitation to dissolve the medium completely. Do not autoclave or over heat. Cool to about 50^0C, mix and pour in to sterile petri plates.

Xylose Lysine Decoxycholate Agar(CDH)

Suspend 55g in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Cool and pour in to sterile petri plates. Do not autoclave or over heat.

SIM Medium (HIMEDIA)

Suspend 36.23g in 1000ml distilled water. Boil to dissolve the medium completely. Dispense 5ml in to screw caped test tubes. Sterilize by autoclaving at 15lbs pressure (121^0C) for 15 minutes.

Rappaport Vassiliadis modified semisolid Medium (Bacto)

Suspend 31.75gm in 1000ml distilled water and boil to dissolve. Do not autoclave. cool to 50^0c. Aseptically add 10ml Bacto Novobiocin anti-microbic supplement; mix well and dispense in test tubes.

MRVP Medium(Buffered Glucose Broth)(HIMEDIA)

Dissolve 17g in 1000ml distilled water. Heat if necessary to dissolve the medium completely. Distribute in test tubes in 10ml amount and sterilize by autoclaving at 15lbs pressure (121^0c) for 15 minutes.

Appendices 2- Procedures of some primary and secondary biochemical tests

Motility test (Quinn *et al*., 2002)

Direct microscopy using young broth culture (2 - 4hours) incubation of the bacterium incubated at room temperature. A " hanging drop " preparation was made by placing a drop of broth culture in the center of a clean cover slip. The preparation is brought in to focus under low power (40x) and reduced illumination and examined for Brownian movement.

KOH test

A loop full of the culture from blood agar and mixed with an equal amount of 3% KOH on a clean microscopic slide, after through mixing the loop is lifted at interval to see whether a gel is formed or not.

Sugar Fermentation broth

Peptone (10 g), Sodium chloride (5 g), Phenol Red (0.018 g)

Distilled water (DW) 1 litre pH: 7.4 ± 0.2

The following sugars, such as dulcitol, glucose, lactose, maltose, mannose, mannitol, sucrose, cellobiose, arabinose, raffinose, trehalose and xylose, and sugar derivatives such as inositol, salicin, sorbitol were used as required. To 100 ml of the basal media, lgm of the respective sugar was added, dissolved, dispensed in 4ml quantities in l00xl2mm tubes containing inverted Durham's tubes. The media was sterilized at 115°C for 20 min. In case of disaccharides and oligosaccharides such as lactose, maltose, sucrose, cellobiose, trehalose and raffinose, filter sterilized solutions were was added to the pre-sterilized broth.

List of *Salmonella* biochemical tests for *Salmonella*

Tests /Media	***Salmonella* typical reaction**
Gram's stain	Gram negative, short rods
TSI	Acid butt, alkaline slant & H_2S +ve
Urease	-ve
Lysine decarboxylase	+ve
Glucose fermentation	Acid & gas
Indole test	-ve
Lactose fermentatiion	-ve
Sucrose utilization	-ve
VP test	-ve
MR test	+ve
Simmons citrate agar	+ve

Maintenance of stock culture

The pure culture of *Salmonella* species were inoculated into the tubes containing TSI slant and incubated at 37^0C for 24 hours. After the growth of organisms, the tubes were sealed with liquid paraffin (light) and kept in the refrigerator at $+4^0$C for further studies.

Appendices 3- Serological test procedures

Rapid serum plate agglutination (SPA) test

Standard *salmonella* antigen manufactured by National Veterinary Institute will be used for Serum Plate Agglutination test. 0.02 ml antigen and 0.02 ml chicken sera were placed side by side with a micropipette on a glass slide and mixed thoroughly by stirring with tooth pick followed by rocking. Results will be read within 2 minutes.

Preparation of somatic antigen

The isolates of *S.* Gallinarum and *S.* Pullorum organism were cultured on SS agar media. Incubated overnight at 37°C and selected a smooth colony and carried out slide agglutination test to ensure that the required somatic antigen is present. A pure culture on nutrient agar slope after incubation for 8-12 hours at 37^oC was washed off the plate by using pasteur pipette with 2 ml of absolute alcohol. It was then transferred into a sterile container. The antigen was left for 4-6 hours at room temperature to enable the alcohol to kill the bacteria. The container was spined for 5 minutes at 1000 rpm. The liquid was poured off and added enough phenol saline to make the antigen up to opacity. Standard titration was carried out with known serum to ensure that the antigen is positive for the required factor and stored at 4°C.

Appendices 4- Some procedures in Molecular characterization

DNA Extraction Procedure using DNeasy Blood & Tissue kit ***(****Qiagen, Hilden, Germany****)***

1. Add 200µl young cultured bacteria at log phase centrifuge at 190rpm.
2. Add 20µl proteinase K and mix by vortexing.
3. Add 200µl buffer aluminum, mix thoroughly by vortexing.
4. Add 200µl ethanol (90-100%) . Mix thoroughly by vortexing.

5. Pipette the mixture in to DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 8,000 rpm for 1 minute discard the follow and collection tube.
6. Place the spin column in new 2ml collection tube. Add 500µl buffer AW1. Centrifuge for 1 min at 6,000rpm discard the follow through collection tube .
7. Place the spin column in to new 2ml collection tube, add 500µl buffer AW2, and centrifuge for 3min at 1400 RPM and the follow through collection tube.
8. Transfer the spin column to a new 1.5ml or 2ml micro centrifuge tube.
9. Elute the DNA by adding 200ml buffer AE2 the center of the spin column membrane, incubate for 1min at room temperature (15-25^{0}C) centrifuge for one min at 8,000 RPM.
10. Optional repeat step 8 for increased DNA yield.

    NB- DNA Extraction was in separate room (DNA extraction room). The DNeasy Blood & Tissue Kit (cat. nos. 69506) can be stored at room temperature (15–25°C) for up to 1 year.

Salmonella Master mix preparation

Type of primer	**Volume required for each rxn**	**Total volume for 16 rxn**
Rnase free water	2µl	32µl
SG-F	2µl	32µl
SG-R	2µl	32µl
SGP-F	2µl	32µl
SGP-R	2µl	32µl
SE-F	2µl	32µl
SE-R	2µl	32µl
Super mix	10µl	160µl
Template	3µl from each sample	
	27µl reaction volume for each sample	

mPCR Reaction

Initial Denaturation 94^0c/5min

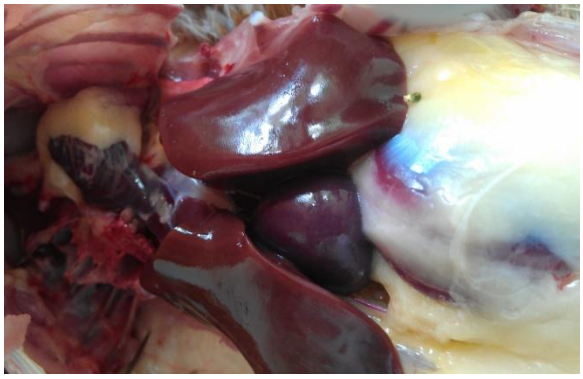
Denaturation = 94^0c/1min
Annealing = 55^0c/1min
Extention = 72^0c/1min
} 35 cycles

Final extention= 72^0c/10min

stored at $+4^0$c until reaction.

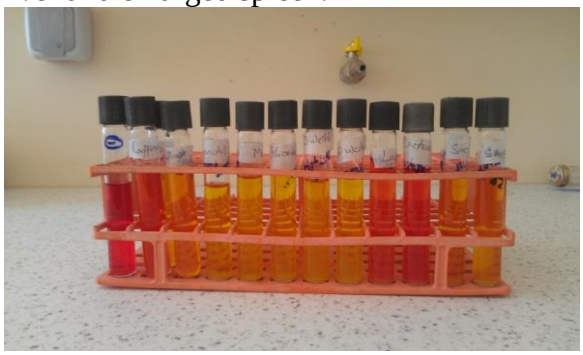
mPCR Reaction was done using thermal cycler (Applied Bio-systems 2720). All procedures were performed in separate room (DNA extraction room, master mix preparation room, PCR room and Gel documentation room) to avoid contamination.

Appendices 5- List of photos captured during laboratory works



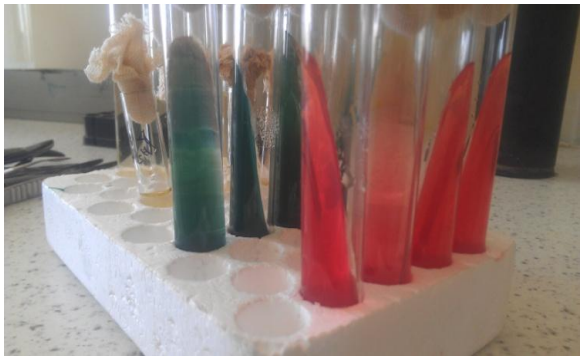
1. *Salmonella* infected chicken showed swollen, shiny and bronze discoloration of liver and enlarged spleen.

2. Day old chicks reared for challenge experiment

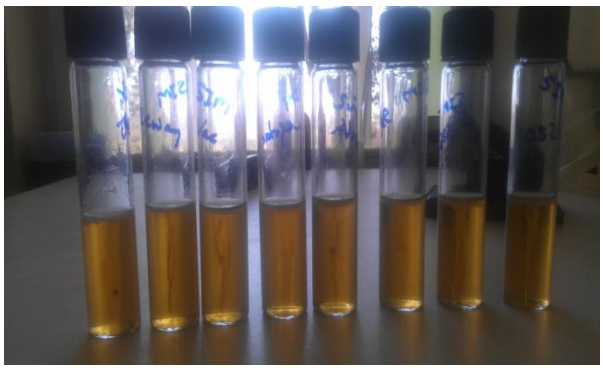


3. Sugar fermentation test results which shows a characteristics of *salmonella* Gallinarum

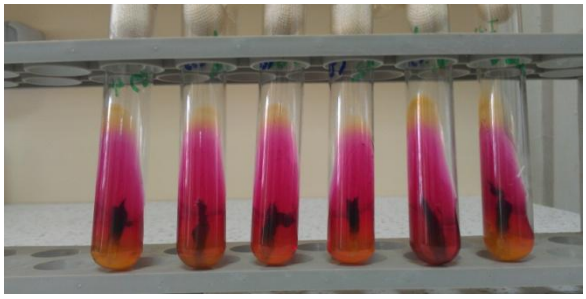
4. Few biochemical test results (TSI, Citrate utilization, Methyl red and vagous proscar)



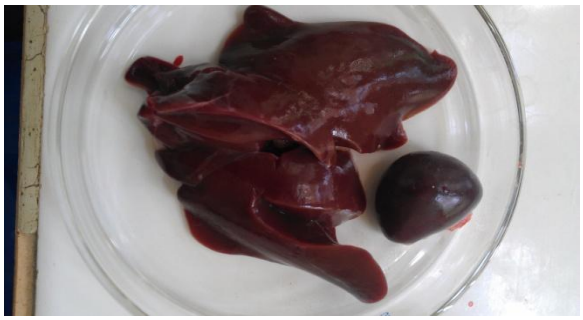
5. Few biochemical test medias used for isolation and identification of Salmonella(MRVP, Indole, Citrate, TSI).



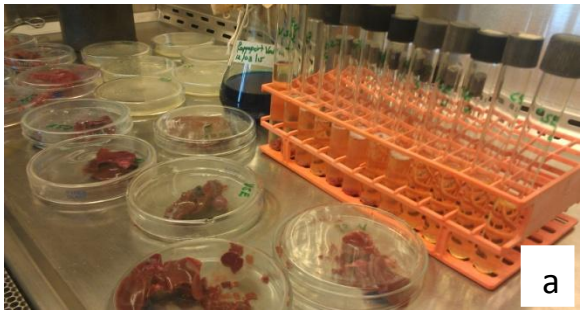
6. *Salmonella* inoculated on SIM media for Motility Test.

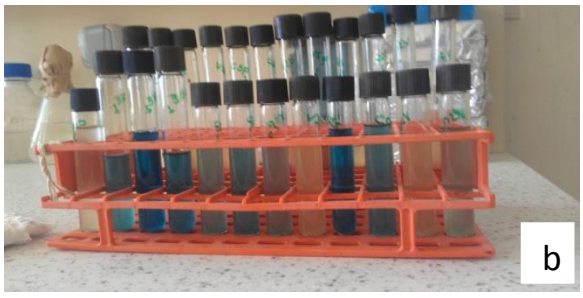


7. Biochemical results of the isolates on TSI after 24hr incubation(R/Y/H_2S).

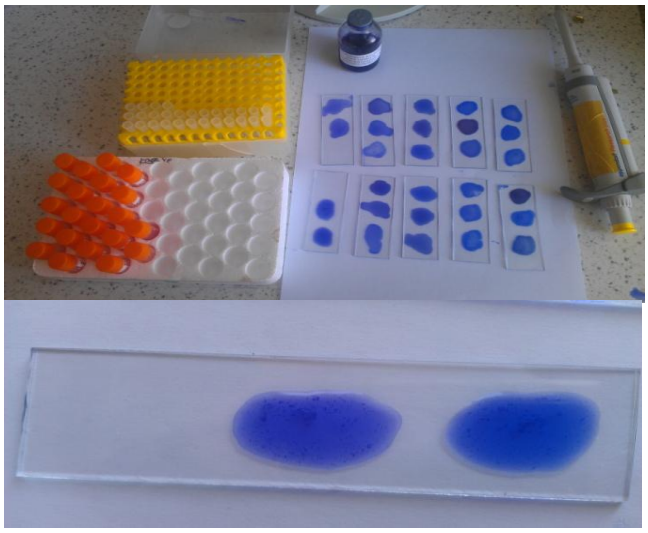


9. Aseptically collected liver and spleen





11.(a) pre-enrichment of pooled 1gm of liver & spleen inoculated in to peptone water. (b). Enrichment stage in Rapapport vaslidies broth for bacterial recovery from internal organs.



8. Detection of antibody by Rapid serum slide agglutination test

10. Bacterial recovery from liver and spleen 14 days after challenge shows typical colonies of *Salmonella* on EMB agar

12. Challenge bacteria preparation and standardizing the turbidity using 0.5 MacFarland standard.

Appendices 6- Clinical Examination Record

<table>
<tr><td colspan="6">Sample Ref. No. _____</td></tr>
<tr><td rowspan="2">Animal Identification</td><td>Species</td><td>Breed</td><td>Sex</td><td>Age</td><td>Production Type</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="7">Case History:</td><td colspan="3">Disease Onset :</td><td colspan="2">No. of Sick:</td></tr>
<tr><td colspan="3">Type and Number Sample Submitted</td><td colspan="2">No. at Risk:</td></tr>
<tr><td colspan="3">1.</td><td colspan="2">No. of Death:</td></tr>
<tr><td colspan="3">2.</td><td colspan="2">Sampling Date:</td></tr>
<tr><td colspan="5">Treatment given:</td></tr>
<tr><td colspan="5">History of vaccination:</td></tr>
<tr><td colspan="5">Sample collection area:</td></tr>
<tr><td colspan="6">Sample preservative used:</td></tr>
<tr><td colspan="6">Clinical Signs Observed:</td></tr>
<tr><td colspan="6">Post Mortem Findings:</td></tr>
<tr><td colspan="6">Suspected Disease/ purpose of test:</td></tr>
<tr><td colspan="6">Recommended/Selected Test/s:</td></tr>
<tr><td colspan="6">Owner’s Name and Signature:</td></tr>
<tr><td colspan="6">Sample submission date:</td></tr>
<tr><td colspan="6">Sample stored at:</td></tr>
<tr><td colspan="3">Sample transferred to:</td><td colspan="2">Signature :</td><td>Date :</td></tr>
<tr><td colspan="3">Request Approved by:</td><td colspan="2">Signature :</td><td>Date:</td></tr>
</table>

Appendices 7- Post Challenge Follow up record

Ref. No. _____			
Species	Breed	Sex	Age
Date of vaccination			
Date of Challenge experiment			
Type of challenge Organism (yes/no)			
		Vaccinated: ☐	
		Control: ☐	
Physical Examination (yes/no) ☐			
			Depression: ☐
Mortality (yes/no) ☐			
Post mortum examination			
		Liver*	
		Spleen*	
Date		Signature	

*0 = no lesion, 1= Mild, 2= medium and 3= severe