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EVALUATION OF PROTECTIVE EFFICACY OF IRRADIATED *SALMONELLA* GALLINARUM VACCINE AGAINST FOWL TYPHOID IN SASSO BREED CHICKEN

MVSc THESIS

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EVALUATION OF PROTECTIVE EFFICACY OF IRRADIATED *SALMONELLA* GALLINARUM VACCINE AGAINST FOWL TYPHOID IN SASSO BREED CHICKEN

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

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Yitbarek Habtamu** entitled: **Evaluation of protective efficacy of irradiated *Salmonella* Gallinarum vaccine against fowl typhoid in Sasso breed chicken** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

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STATEMENT OF AUTHOR

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TABLE OF CONTENTS

CONTENTS **PAGES**

LIST OF ABBREVIATIONS

ALIPB	Aklilu Lemma Institute of Pathobiology
BHIA	Brain Heart Infusion Agar
BIHB	Brain Heart Infusion Broth
CFU	Colony Forming Unit
CVMA	College of Veterinary Medicine and Agriculture
DNA	Deoxyribonucleic Acid
ID_{50}	Infectious Dose 50
LD_{50}	Lethal Dose 50
ELISA	Enzyme Linked Immuno-Sorbent Assay
FT	Fowl Typhoid
IB	Infectious Bronchitis
KGy	Kilo Gray
NAHDIC	National Animal Health Diagnostic and Investigation Center
NICETT	National Institute for Control and Eradication of Tsetsefly and Trypanosomosis
NVI	National Veterinary Institute
OD	Optical Density
OIE	International Organization for Animal Health
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD	Pullorum Disease
Rpm	Revolution per minute
RV	Rappaport Vassiliadis
S/C	Subcutaneous
SG	*Salmonella* Gallinarium
SAT	Slide Agglutination Test
SPF	Specific Pathogen Free
VDFACA	Veterinary Drug and Feed Administration and Control Authority

LIST OF TABLES **PAGES**

LIST OF FIGURES **PAGES**

LIST OF ANNEXES **PAGES**

ABSTRACT

Fowl typhoid is worldwide distributed septicemic disease of chicken, turkeys, ducks, pheasants, guinea fowl, peafowl, sparrow, goose, and quail caused by *Salmonella enterica* subspecies enterica serovar Gallinarum. Live attenuated 9R strain of *Salmonella* Gallinarum (SG) is commonly used vaccine for the control of fowl typhoid. However, its persistence in vaccinated chickens causes vertical transmission through eggs and the residual virulence inducing lesions in the liver and spleen in some breeds of chicken are the drawbacks of this vaccine. In recent vaccine development efforts, alternative methods to develop a variety of vaccine types have been attempted of which radiation inactivated pathogens are some of them. Irradiation can avoid chemical contaminants from chemical inactivation and penetrate pathogens to destroy nucleic acids without damaging the pathogen surface antigens. The objective of this study was to evaluate protective efficacy of gamma irradiated *Salmonella* Gallinarum vaccine against fowl typhoid in poultry. After the strain identification test, inoculum of approximately 10^9 cfu/ml of field strain of *Salmonella* Gallinarum was prepared and exposed to series of radiation dose ranging from 1.1-3 kilo gray (kGy) for inactivation of which 2.6 KGy was found to be minimum lethal dose and it was used for final irradiation dose in this study. Forty two (42) days old Sasso breed of chickens were allocated randomly to six groups having 10 chickens each: G_1 (vaccinated with irradiated *Salmonella* Gallinarum vaccine but not challenged), G_2 (vaccinated with irradiated *Salmonella* Gallinarum vaccine and challenged), G_3 (vaccinated with irradiated *Salmonella* Gallinarum vaccine, provided with booster dose 21 days later and challenged), G_4 (vaccinated with irradiated *Salmonella* Gallinarum containing 20% trehalose and challenged), G_5 (vaccinated with 9R commercial *Salmonella* Gallinarum vaccine produced at NVI, Bishoftu, Ethiopia, and challenged) and G_6 (unvaccinated but challenged group as a control). Prior to the immunization process, all chickens were assessed for the presence of antibody against *Salmonella* Gallinarum on the 7^{th} weeks of age and none of them were found serologically positive using slide agglutination test (SAT). The homologous challenge infection experiment was conducted using the standard field dose of (~5.3 x 10^7 cfu/ml) ~ with optical density value of 0.6. On day 21, G_4 and G_5 showed strong antibody production than other groups, 80% and 90%, respectively. Fifty (50%) of G_1 showed strong antibody production and 50% of them

moderate reaction. However, only 20% and 30% of G_2 and G_3 respectively produce strong reaction on day 21 post vaccination. As G_6 was unvaccinated group, there was no reaction throughout the experiment. But G_3 on booster dose after 2 weeks of vaccination showed 60% strong agglutination on day 35 of first vaccination. Up on the challenge, chickens in G_3 and G_4 showed significant difference in survival rate (70%) over G_2 and G_6 which only 20% of them survived. Survived and sacrificed chickens at the end of experiment showed significantly lower lesions and bacterial re-isolation from the liver, spleen and gizzard as compared to those birds died during challenge experiment. There was significantly higher number of survivors among vaccinated G_3 & G_4 as compared to non-vaccinated group (G_6) ($p<0.0001$). There was significant difference ($p<0.05$) in level of protection between G_2 and G_4 as well as between G_2 and G_5. Survivors in G_5 were 100% that showed commercial 9R vaccine conferred strong protection as compared to G_2, G_3 and G_4 and their protection was 20%, 70% and 70% respectively. There was no significant level of protection in chickens in G_2 compared to unvaccinated control group ($p>0.05$). Addition of 20% trehalose and booster dose improved protection of irradiated vaccine by50%. In conclusion, subcutaneously administered irradiated SG candidate vaccine with 20% trehalose and booster dose of irradiated vaccine without 20% trehalose showed promising safety, immunogenicity and protective efficacy. Further studies on safety, shelf life, radiation dose optimization for trehalose added irradiated *Salmonella* Gallinarum vaccine and quantification of antibody response using ELISA and other immunological methods are recommended.

Key words: Fowl typhoid, Irradiated vaccine, *Salmonella* Gallinarum, Trehalose, 9R vaccine,

1. INTRODUCTION

The etiological agent of fowl typhoid is *Salmonella enterica* subspecies *enterica* serovar Gallinarum biovar Gallinarum (Shivaprasad, 2000; Vaid *et al.,* 2015) which is found throughout the globe, dominantly in developing countries (Barbour *et al*., 2015). Fowl typhoid cause by *Salmonella* Gallinarum recurred in some countries of Europe in the 1st decade of 21st century (Ivanics *et al*., 2008). There is a record of fowl typhoid eradication in commercial poultry farms of many developed countries like USA, UK, Australia, Canada, Western Europe and Japan (Shivaprasad, 2000). The serovar is known as *Salmonella enterica* subspecies *enterica* serovar Pullorum in UK and United States of America (Hitchner, 2004), though it is confirmed that the strain is similar with the serovar derived from *Salmonella enterica* by the gene deletion process (Thomson *et al*., 2008).

Vaccines are most effective in protecting populations at risk, which typically affected by varied geographical settings (Revolledo, 2018). For live vaccines to survive, the logistical complexities of mass vaccination campaigns, a maximally stable product are required (OIE, 2018). Among pharmaceuticals, vaccines have the greatest need for stability and yet they are among the most difficult to stabilize, leading to a significant challenge for the vaccine developers (Plotkin and Plotkin, 2011). Some mutant strains were tested to replace SG vaccine; however, they are not commercially available (Harbourne *et al*., 1963; Revolledo and Ferreira, 2012; Lee, 2015).

Vaccination with live 9R strain vaccine may sometimes precipitate high mortality in vaccinated birds because of its unknown level of attenuation (Silva *et al*., 1981; Thomson *et al*., 2008).

Even though it is not an easy task to identify vaccinal (commercial) strains from the field strains of *S*. Gallinarum, they should be distinguishable strains and commercial vaccines are recommended to use to maintain quality and avoid the risk of reversion and virulence (Van Immerseel *et al*., 2013).

Vaccination helps the development of naturally acquired immunity by inoculation of non-pathogenic but still immunogenic component or closely related organism (Meeusen *et al*., 2007). However, the rough *Salmonella* Gallinarum 9R strain vaccine, most widely used has a number of drawbacks such as: immunized chickens may transmit to eggs, and the residual virulence inducing lesions in the liver and spleen (Silva *et al*., 1981). These drawbacks led researchers and scientists to find other possible ways of developing novel effective vaccines (Barrow *et al*., 2012). Radiation inactivation has been used to develop a variety of vaccine types, because of its ability to eradicate chemical contaminants and penetrate pathogens to destroy nucleic acids without damaging the pathogen surface antigens (Seo, 2015).

In Ethiopia, live attenuated 9R strain of *Salmonella* Gallinarum vaccine which has been used for long period to control fowl typhoid is available. Due to selective pressure from targeting to specific serovar of *Salmonella* Gallinarum for vaccination, population shift has been reported in *Salmonella* serovars which needs regular monitoring of protective efficacy of the vaccine in use (Foley *et al*., 2011). Therefore, the objectives of this study are: -

General objective

- To evaluate immunological and protective efficacy of irradiated *Salmonella* Gallinarum vaccine for the control of fowl typhoid in poultry.

Specific objectives

- To establish optimum doses of Gamma irradiation which results in a complete inactivation of *S.* Gallinarum and assess it immunogenic potential in chicken.
- To assess the protective efficacy of the irradiated SG vaccine against homologous challenge in comparison with commercial strain 9R vaccine in Sasso breed chickens
- To assess the role of irradiating *S.* Gallinarum with trehalose (20%) on protective efficacy of vaccine
- To investigate the role of booster dose of irradiated SG vaccine in improving protection efficacy
- To assess and compare the survival rate and level of pathogenicity based on the occurrence of pathological lesion as marker of efficacy of protection of irradiated SG vaccine

2. LITERATURE REVIEW

2.1. Fowl Typhoid

Fowl typhoid (FT) is an infectious disease of domestic birds that occurs sporadically or enzootically in most countries in the world including Ethiopia**.** The causative agent of FT is *Salmonella enteric* sub species *enterica* Serovar Gallinarium biovar Gallinarum (Kang *et al.,* 2012; Ahamed *et al.,* 2017). *Salmonella* Gallinarum belongs to the family Enterobacteriaceae. It is a non-spore forming, non-capsulated, gram-negative plump rod, *Salmonella* belonging to serogroup D. It is short non-flagellated organisms with O antigens 1, 9 and 12 (Shivaprasad, 2000), capable of producing septicemic disease in domestic and wild birds (Gong *et al*., 2016). *Salmonella* Gallinarum, primarily causes disease in chickens and turkeys in many countries (Kang *et al*., 2012) but ducks, pheasants, guinea fowl, peafowl, sparrow, goose, and quail can also be affected (Shivaprasad, 2000). The first Asian region draft genome sequence of isolated *S.* Gallinarum strain from the liver of a broiler chicken (*Gallus gallus domesticus*) in India and its whole-genome sequence has been deposited at gene bank under the accession number JSWQ00000000 (Vaid *et al*, 2015). Latter on other strains of *Salmonella* Gallinarum strains like SG9, 287/92, FCAV198, CDC1983-67, 9184, and RKS5078 have been sequenced from North America, Brazil, and the United Kingdom (Vaid *et al.,* 2015).

2.1.1. Global distribution of fowl typhoid

Fowl typhoid is widely distributed disease throughout the world except countries such as the U.S., Canada, New Zealand, Australia, Japan and other countries in Western Europe eradicated from commercial poultry in nations with highly developed poultry industries (Kumar *et al*., 2010). However, *Salmonella* Gallinarum may persist in backyard flocks, game and wild birds of these countries. Fowl typhoid is common in some countries of Eastern Europe *e.g*. Romania and Poland, Central and South America, Africa and Asia including Ethiopia (Kumar *et al*., 2010; Shivaprasad, 2000; Ashenafi *et al*., 2003).

2.1.2. Epidemiology of fowl typhoid in Ethiopia

Poultry production is an integral part of animal production which improves the livelihood of people living in the rural areas of developing countries (Pagani and Wossene, 2008). In Ethiopia, the total poultry population is estimated to be 50 million (Pagani and Wossen, 2008). Now a days it is very common to see commercial poultry farms in and around the urban areas of Ethiopia (Pagani and Wossene, 2008). However, the sector is not generating economic assets as expected. This is because of different reasons among which unimproved breeds, disease and primitive management systems are the major factors (Dinka *et al*., 2010).

According to Tadele *et al.* (2014), the overall sero-prevalence of Fowl typhoid in chicken at Eastern part of Ethiopia, was 30.7% and prevalence in Dire Dawa was 32.5% and 38.6% for the commercial and the local breeds, respectively. Seroprevalence in different parts of Ethiopia like Addis Ababa, eastern and central Ethiopia and Mekelle ranges from 10.44% to 64.2% (Ashenafi *et al*., 2003; Berhe *et al*., 2012). Morbidity, mortality and fatality rates of 69%, 67.7% and 97% respectively in the newly introduced flock; and 5.75%, 5.5% and 96% in the previous existing flocks were observed in Jimma Agricultural College farm (Abie *et al*., 2003).

In Ethiopia, the current rates of mortality due to different poultry diseases from day old to adult chicken are estimated to be 20-50% (Aragaw *et al*., 2010 ; Endris *et al*., 2013). Among the diseases; fowl typhoid is mentioned to cause heavy losses (Endris *et al*., 2013). The study by Berihun *et al.*(2012) in local and exotic chickens of commercial poultry farms of Ziway and Debre Zeit also indicated that large amount of backyard (local chickens) (44.6%) were affected by fowl typhoid compared to those chickens kept intensively with the prevalence of 26.6% (Table 1).

Immunization and strong bio-security is considered to be very useful mechanism in controlling the disease introduction in to the poultry farms since the disease is endemic to the country. The vaccine that has been used against the disease and available for long time in Ethiopia is a live attenuated vaccine of *Salmonella* Gallinarum 9R strain (Adamu *et al*., 2017).

Table 1: Sero-prevalence of *Salmonella* Gallinarium in some parts of Ethiopia

Study Area	Farming system/Breed	Sample size	Sero Positive	Prevalence %	References
Haramaya University	Chicken	309	95	30.7	(Tadele *et al*., 2014)
Dire Dawa university	Commercial	40	13	32.5	(Tadele *et al*., 2014)
	Local	44	17	38.6	
Ziway and Debre Zeit	Back yard	419	187	44.6	(Berihun *et al*., 2012)
	Intensive	395	105	26.6	
Debre Zeit	Local	100	11	11	(Endris *et al*., 2013)
	Exotic	50	3	6	
Tigray and Addis Ababa	Commercial-local	279	55	19.71	(Kumar *et al*., 2014)

2.1.3. Transmission

Fowl typhoid can be transmitted through oral route from contaminated food and water or by cannibalism and via the respiratory tract (Shivaprasad, 2000). The causative organisms may also enter the body at other sites, such as in wounds (Hall *et al*., 1949). Sick bird is the most dangerous spreader of fowl typhoid and the carrier bird is probably the most important perpetuator of the disease, improper disposal of dead chickens is one of the greatest factors in spreading and perpetuating fowl typhoid in a community (Gordeuk *et al*., 1949; Hall *et al*., 1949). Under optimal conditions, *Salmonella* Gallinarum may survive for several months and possibly up to several years in the environment (Griffin and Barrow, 1993). However, they can be destroyed by sunlight and/or high environmental temperatures, and they may only survive for a few weeks or less at some sites. Wild birds, mammals and insects can act as mechanical or biological vectors (Shivaprasad, 2000; Wales *et al*., 2010).

2.1.4. Clinical signs

Clinical signs include: drooping of wings, loss of appetite, reluctance to move, increase in water consumption, depressions, weight loss, dehydration, ruffled feathers, watery to mucoid yellowish diarrhea, respiratory distress and dead chicks are common clinical signs in poultry and game birds (Shivaprasad, 2000). Egg production usually decreases,

decreased hatchability and fertility may be noted, a progressive loss of condition in birds that survive longer can lead to anemia with pale, shrunken combs and wattles (Shivaprasad, 2000). There may also be outbreaks with atypical syndromes characterized by decreased egg production and high mortality in quils, but few birds may not show any clinical signs. Fowl typhoid has per acute, acute or chronic form of disease affecting mostly adult chickens (Endris *et al*, 2013; Ahamed *et al*., 2017).

2.1.5. Morbidity and mortality

Fowl typhoid is characterized by septicemic disease often seen in growing birds and adults (Thomson *et al*., 2008), although chicks are susceptible. The mortality rate is reported to range from 1% to 100% in chickens and turkeys, with morbidity and mortality affected by the age, species and breed of the bird; flock nutrition and management; stressors including concurrent infections; and the strain of the organism (Shivaprasad, 2000).

2.1.6. Prevention and control

Developing countries have programs for controlling, preventing, and eradicating Fowl typhoid but the legal framework is not updated according to new approaches, the epidemiological situation, or new tools for diagnosis or prevention (Revolledo and Ferreira, 2012).

To exclude *S.* Gallinarum from a poultry flock, live birds and eggs should be purchased from stock known to be free of these organisms, or tested (Shivaprasad, 2000). Good biosecurity is also important in excluding organisms that may be present on fomites and visitors, rodents and wild birds should be excluded from the facility and potential insect vectors and/or reservoirs, infected flocks are usually quarantined in fowl typhoid free countries so that repeated testing and removal of carriers can sometimes eliminate the infection from a flock (Parvej *et al*., 2016; Lee, 2015).

More often, the entire flock is depopulated and the premises are cleaned and disinfected before restocking (Gordon and Luke, 1959). Vaccination can protect birds from clinical signs and mortality, but it does not prevent them from becoming infected, and

protection may be short-lived (Gordon and Luke, 1959). It is difficult to eliminate *S.*Gallinarum from game birds in semi-wild production systems where the birds are released into the wild and recaptured for breeding (Shivaprasad, 2000).

2.1.7. Public health importance

This bacterium limits itself to avian species and is not known to cause disease in humans; however, there is zoonotic potential of rarely causing clinical cases in people. In one report, out of more than 450,000 *Salmonella* isolated from humans, 26 were found *Salmonella* Gallinarum in the U.S. between 1982 and 1992 and urinary tract infection caused by *Salmonella* Gallinarum was reported in a kidney transplant patient in 2017 (Ahamed *et al*., 2017). Self-limiting enteritis is thought to be the most common presentation, although other syndromes have been reported and their threat to immunocompromised individuals is still unclear (Ahamed *et al*., 2017; Eswarappa *et al*., 2009).

2.2. Vaccine Development

2.2.1. Biological characteristics of conventional fowl typhoid vaccine

For the strain to be used as a vaccine, it should be close related to the field strain bacteria which is recently circulating, it should be identified from severe clinical cases, its antigenic property, virulence, stable phenotypic and genetic markers should be checked and the whole genome sequencing is recommended (de Paiva *et al*., 2009; Van Immerseel *et al.*, 2013; Pujar *et al*., 2015; OIE, 2018).

Whole genome sequencing can be used to check and verify the stability of the live vaccinal strain of the vaccine regularly (OIE, 2018). As the 9R *Salmonella* Gallinarum is derived from *S. enterica* by gene deletion process, other live vaccines can also be produced from one or more strains with metabolic drift attenuation via an increased generation time (Linde,1982; Thomson *et al*., 2008).

2.2.2. Fowl typhoid vaccine development

The 9R vaccine with semi-rough lipopolysaccharide structure is formulated in 1950s (Smith, 1956; Gordon and Luke, 1959). *Salmonella* Gallinarum 9R vaccine strain still results in systemic disease, and it was suggested that variants can cause some outbreaks (Revolledo and Ferreira, 2012). Although the 9R vaccine is considered to be the highly attenuated vaccine as compared to the parental O9 strain, it may still cause disease and pathological lesions in organs like liver and spleen with the bacterial residue in these organs for weeks, especially in young chickens (Feberwee *et al*., 2001; Wigley *et al*., 2005). Therefore, it is difficult for veterinary clinicians to know whether *Salmonella* Gallinarum in the affected chicken, especially in those with under 2 weeks of age is from pathogenic field isolates or the 9R vaccine strain based on routine biochemical tests (Lee *et al*., 2005). *Salmonella* Gallinarum strain 9R vaccine has been one of the most popular live vaccine in poultry developed by the William Smith (Barrow *et al*., 2012). The rough strain provides sufficient immunity in chicken, without showing pathogenicity for day old chicks (Feberwee *et al.*, 2001). Drop of egg production and mortality reported with smooth strain was not reported with rough strain in chicken; furthermore smooth strain cause mortality in day old chicks and marked drop in egg production in layers (Barrow*et al*., 2012).

A suspension of live attenuated 9R *Salmonella* Gallinarum vaccine for fowl typhoid has the same biochemical characteristics with field strain of *Salmonella* Gallinarum. A 24 hour grown colonies of *Salmonella* Gallinarum taken from live vaccine using the nutrient agar plate are found rough when observed by acriflavine slide test and it should not reveal characteristics of the smooth forms *S*. Gallinarum colonies (OIE, 2018).

2.2.3. Minimum quality requirements of conventional fowl typhoid vaccines

***Sterility and purity*:** Gram staining using bacterial suspension, vaccine culture on general media to know the homogeneity, biochemical tests, identification of the phenotypic and genetic markers, slide agglutination tests using specific antiserum and checking for the microbiological sterility and non-toxicity of preservatives, adjuvants and other packaging materials are some of the methods to be used for the sterility and purity assessment the vaccines (Pujar *et al*., 2015; Revolledo and Ferreira, 2012).

Safety **:** The 50% lethal dose (LD_{50}) or 50% infectious dose (ID_{50}), sign of adverse reactions in the target species, stability and virulence after repeated passage in susceptible host should be checked (Silva *et al.*, 1981; OIE, 2018). Live vaccinal strains should not show bacterial persistence in immunized chickens, should not be the cause for vertical transmission through eggs (OIE, 2018). At least six healthy chickens , susceptible and preferably specific pathogen free (SPF), at the age of 8-16 weeks are injected subcutaneously with ten-fold dose of *S.*Gallinarum vaccine followed by the minimum of 7 days observation for its adverse reactions (Pujar *et al*., 2015 ; Revolledo and Ferreira, 2012).

In one safety trial of *S.* Gallinarum vaccine, birds were euthanized at six weeks post vaccination; necropsies were performed and samples were taken and tested. No abnormal clinical signs or mortalities were observed either in the group of seven-day old chicks of the safety group, which received one dose of vaccine or in the six week-old birds receiving ten doses at different routes of inoculation of the vaccine (OIE, 2018).

Vaccine stability**:** In killed types of vaccine the stability information is lacking. However, the storage condition, presence of growing contaminant microorganisms in the vaccine suspension and absence of preservative which have antimicrobial activities ,like phenol/crystal violet or thiomersal affected the stability (Pujar *et al*., 2015; OIE, 2018). Even though there is lack of information for the stability of killed vaccine, viable cell count and potency tests repeated at appropriate time interval and genotyping test during fermentation can be performed to assess the stability of live vaccines (Revolledo and Ferreira, 2012; Pujar *et al*., 2015).

Efficacy**:** To evaluate that the vaccine is effective or not, laboratory experiments and field trial should be conducted. Even though the vaccination-challenge experiments are possible in the laboratory at recommended dose and age, efficacy test in the field is more difficult due to the difficulties in standardizing the challenge and controls (Pujar *et al*., 2015; OIE, 2018). At least fifteen healthy chickens, at the age of 8-16 weeks, brown egg layers, free of *S.* Pullorum are injected subcutaneously with viable cells corresponding to one field dose (5×10^7) to evaluate the efficacy of *S.* Gallinarum and similar vaccines (OIE, 2018; Pujar *et al.*, 2015).

***Immunization*:** Immunity to *Salmonella* is serovar specific (Smith, 1956). Duration of the protection of vaccine (immunity) depends on the type of the product (live or killed vaccine), regimes of vaccination, nature of the vaccinated animals (Pujar *et al*., 2015). Vaccines should protect chickens throughout their laying period. A booster dose of the *Salmonella* Gallinarium vaccine against fowl typhoid should not be provided during the laying period because of the vertical transmission nature of the 9R attenuated live vaccines (Pujar *et al*., 2015; OIE, 2018). Some live vaccines administered by injection may develop stronger immunity which may stay for one year or more, while most killed vaccines elicit six months of protection (Plotkin and Plotkin, 2011; Pujar *et al*., 2015).

2.2.4. Current status of fowl typhoid vaccine development

There is on-going interest in finding ways to prevent and control flock infection. In developing countries where *S.* Gallinarum is endemic and new attenuated strains are being investigated, vaccination is considered as the best strategy for the control of Fowl typhoid (Griffin and Barrow, 1993). Inactivated products are killed bacteria, complemented with different adjuvants to improve their immunogenic properties; however, they do not elicit a cell-mediated immune response, which is indispensable for the clearance of *Salmonella* Gallinarum (de Paiva *et al*., 2009).

The use of *Salmonella* Gallinarum 9R in poultry outbreaks in the field in clinically healthy birds has a high frequency of increasing the virulence of the *Salmonella* Gallinarum 9R strain, which might be explained by the interaction among the circulating strain, the vaccine strain, and the host; certain environmental conditions also may be involved in the reversion of the virulence of the vaccine strain (Gordon and Luke, 1959). Later, many attempts to prevent FT by vaccination with *S.* Gallinarum strains were developed; some mutant strains were tested, but they are not commercially available, except for the *Salmonella* Gallinarum 9R strain (Revolledo, 2018).

As a safe alternative, an attenuated live vaccine containing a metabolic drift mutant strain of *S.* Enteritidis was used with success against fowl typhoid in layers (Chacana and Terzolo, 2006).

In 2000, an aromatic dependent (*aro*A) mutant wild-type *S.* Gallinarum strain that was made lysogenic for P22 sie was developed (Barrow *et al*., 2000) and it was the first report of attenuation associated with lysogenization. It was administered intramuscularly and had excellent results in immune protection studies, conferring 100% protection against the homologous strain (Barrow *et al*., 2000).

In 2007, a *met*C mutant of *S.* Gallinarum was constructed, and various experiments were carried out to assess the effects of this mutation on virulence and invasiveness that suggested the *met*C mutant of *S.* Gallinarum could be a potential genetically engineered vaccine candidate against fowl typhoid (Shah *et al*., 2007). Another mutant of *S.* Gallinarum was developed and evaluated in Brazil with deletions in genes *cob*S and *cbi*A, which are involved in the biosynthesis of cobalamin, and this mutant strain was tested for efficacy in 2 experiments performed separately and administered once or twice and showed efficacy in brown chickens against mortality caused by the *S.* Gallinarum wild-type challenge (Revolledo, 2018).

2.2.5. Radiation and vaccines development

Radiation is the emission or transmission of energy in the form of waves (ionizing radiation) or electron particles (non-ionizing radiation) (Delaney *et al*., 2005). The use of ionizing radiation, including X-rays and gamma rays, has increased substantially over the last 30 years in both medicine and industry (Amin *et al.*, 2014). Radiation is used as a safe and convenient method of sterilization worldwide (Begun *et al*., 2011). Radiation technology has also been used in the development of human and animal vaccines, especially in the sterilization and generation of random mutations that many vaccines used today rely on technologies developed over 100 years ago, and involve some form of attenuation (*i.e*., the use of an alternative or mutant strain of pathogenic organism with reduced virulence that maintains its immunogenicity, or inactivation, where chemical or physical methods are used to kill virulent pathogenic strains and protecting against animal and human diseases caused by viruses and bacteria) (Seo, 2015).

Radiation technology is of interest to vaccine manufacturers, because it can remove chemical contaminants and penetrate pathogens to damage the DNA (Seo, 2015). Alternative methods to chemical inactivation include radiation technologies; Ultraviolet light was shown to damage not only nucleic acids but also proteins, hence its application in vaccine development is challenging that most prominently, gamma or high-energy electron radiation is being used for pathogen inactivation that ionizing radiation has the advantages of working very quickly and under conditions used for vaccine development; it preferentially damages nucleic acids rather than proteins, resulting in inactivated pathogens that are highly versatile to elicit effective immune responses (Amin *et al*., 2014; Seo, 2015).

2.2.6. Attenuated and inactivated vaccines

Attenuated vaccines in contrast to those produced by "killing" the organism (inactivated vaccine); possess a mutation that enables it to infect the new host, spread or bacteria to grow well in the new host; the result is a population that is significantly different from the initial population, and thus will not grow well in the original host when it is re-introduced (hence it is attenuated) (Parvej *et al*., 2016). This process is known as "passage" in which the pathogen becomes so well adapted to the foreign host that it is no longer harmful to the vaccinated subject (OIE, 2018). This makes it easier for the host's immune system to eliminate the agent and create the immunological memory cells which will likely protect the patient if they are infected with a similar version of the bacterial strain in the field (Parvej *et al*., 2016; OIE, 2018).

Whole bacterial vaccines to be used known as killed or inactivated (OIE, 2018), is fully destroyed using heat, chemicals (Formalin is common vaccine inactivation means), or radiation, Split virus/bacteria vaccines are produced by using a detergent to disrupt the organism (Plotkin and Plotkin, 2011).

2.2.7. Irradiated bacterial whole cell vaccines

Since the typhoid fever vaccine was first introduced as an inactivated bacterial vaccine at the end of the 19th century, the administration of inactivated whole cell bacterial vaccines is one of the well-studied methods of vaccination against bacterial infections (Plotkin and Plotkin, 2011). This approach offers several advantages. First, they are naturally occurring micro particles that can carry multiple antigens that can be important in providing protection. Second, this approach is relatively quick and inexpensive to manufacture (Seo, 2015).

Overall, gamma-irradiation represents a very promising approach for development of highly effective vaccines (Alsharifi and David, 2017). Moreover, the metabolically active forms of irradiated vaccines were able to activate cytotoxic T-cells, which are important immune cells for treating intracellular pathogens. Therefore, radiation inactivation might provide a feasible, broad-spectrum, simple, and effective technique for the development of novel vaccines (Seo, 2015) (Table 2 and Figure 1).

Table 2: Effects of ionizing radiation on different pathogenic Enterobacteriaceae

Culture	Initial Count (CFU/ ml)	Radiation Dose (kGy) and Bacterial Counts (CFU/ml)					
		0.5	1	1.5	2	2.5	3
S. Typhi	10^8	10^5	10^3	10^1	ND	ND	ND
S. Typhimurium	10^{10}	10^6	10^4	10^3	ND	ND	ND
S. Enteritidis.	10^{10}	10^7	10^4	10^3	10^1	ND	ND
S. flexneri	10^8	10^3	10^1	ND	ND	ND	ND
E. coli O157	10^9	10^4	10^2	ND	ND	ND	ND
V. cholera	10^{10}	10^6	10^2	ND	ND	ND	ND

ND=None Detected; Source: (Gelli *et al.*, 1997)

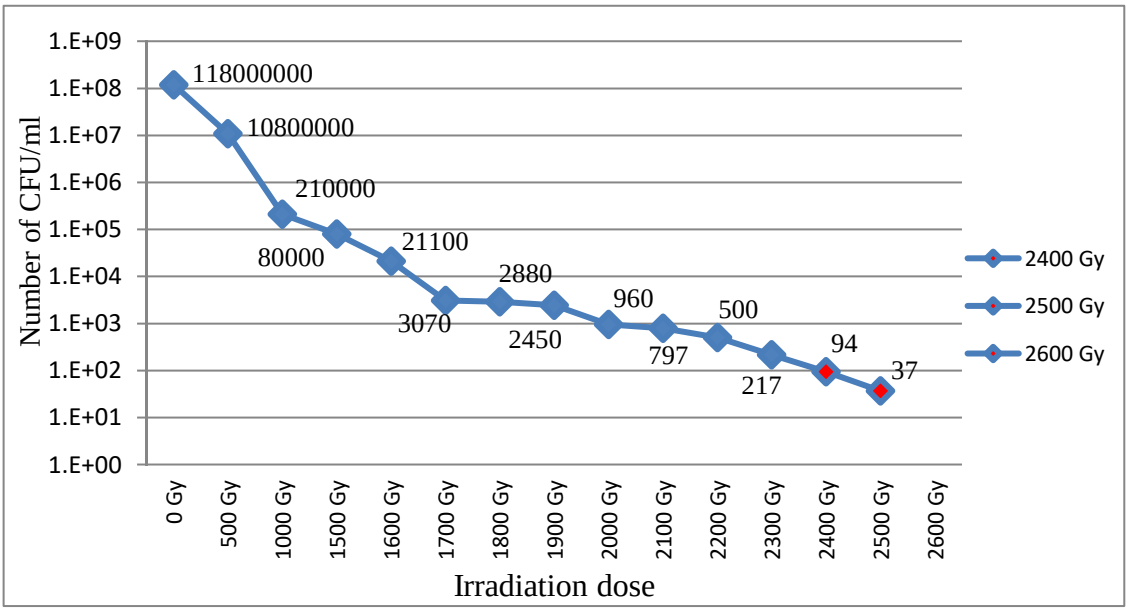

Figure 1: Effect of gamma radiation on *Salmonella* Gallinarum (Lulie *et al*., 2020)

2.3. Fowl Typhoid Vaccine in Ethiopia

Currently, the only freeze-dried form of 9R live attenuated *S.* Gallinarum vaccine against fowl typhoid is produced in National Veterinary Institute (NVI, Bishoftu, Ethiopia). Each vial contains $5x10^7$ CFU of *Salmonella* Gallinarum strain, it is presented in 5ml or 20ml vials of 100 doses, which is recommended to store at -20^{o}C (NVI, 2021). The vaccine is also administered by subcutaneous injection in to the lower part of the back of the neck. The dosage volume was 0.2 ml and the age for first vaccination is 6 weeks, with re-vaccination at 12 weeks of age, it is not recommended for layers (NVI, 2021).

Since 1920s, formalin inactivated vaccines have been used widely. However, the protective efficacy of the vaccine against the disease is limited due to the emergence of new subtypes and strains (Sugaya *et al*., 1994). Even though the use of live vaccine is highly limited due to the difficulties in its development and virulence, it is being used as an alternative measure (Rubin *et al*., 2013). The live attenuated 9R *Salmonella* Gallinarum vaccine against fowl typhoid has been reported to cause unpredictable invasive infection (Silva *et al*., 1981).

Immunization of chickens and strong bio-security is considered to be very useful mechanism in controlling the disease introduction in to the poultry farms (Tadesse *et al*., 2018). The vaccine that has been used against the disease and available for long time in Ethiopia is a live attenuated vaccine of Salmonella Gallinarum 9R strain (Adamu *et al*., 2017).

According to Adamu *et al.* (2017), sero-positivity of chickens vary from 80% at 1st week of post vaccination to 95.6% two weeks post vaccination. However, the selective pressure from using a specific serovar for several years resulted population shift in *Salmonella* serovars (Foley *et al*., 2011).

Regular assessment of the protective efficacy of the vaccine in use is very important to know whether the vaccinal strain is antigenically similar with the recently circulating field strain or not (Foley *et al*., 2011). *Salmonella* Gallinarum 9R strain vaccine showed significant protective efficacy against challenge with field strains of *Salmonella* Gallinarum (Adamu *et al*., 2017).

The rough strain (9R) originated from the smooth strain of 9S that differ in; rough (9R) strain has no the somatic antigen characteristics of the smooth forms of *Salmonella* Gallinarum due to the loss of some lipopolysaccharide. The change in lipopolysaccharide reduced the virulence of the strain (Dal Berrto *et al*., 2015; Van Immerseel *et al*., 2013).

The antibody response of birds vaccinated with conventional and combined vaccines (Lasota strain of Newcastle disease virus (NDV), the FP strain of fowl pox virus (FPV) and the 9R strain of FT) was very effective (Tadesse *et al*., 2018).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted from November 2019 to October, 2021 at Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University and National Institute for Control and Eradication of Tsetse Fly and Trypanosomosis (NICETT), Tsetsefly Mass Rearing and Irradiation Center, Addis Ababa.

3.2. Bacterial Strain Determination

Salmonella Gallinarum field isolate obtained originally from National Animal Health Diagnostic and Investigation Center (NAHDIC) was obtained from ALIPB Microbiology Laboratory and further sub-culturing was conducted by inoculating the in 5ml of Nutrient broth, Tryptic soya broth and Brain heart infusion broth and Nutrient agar, Tryptic soya agar and Brain heart infusion agar to know which medium favours the growth of the organism. Petridishes of 20 ml and the falcon tubes of 5ml broth containing *Salmonella* Gallinarum were incubated at 37°C for 24hrs. Prior to the experiment, bacterial growth condition, colony characteristics, viability and purity were checked. Bacterial growth was followed by purification through sub-culturing. Refreshed colony was observed for colony characteristics and morphology under microscope.

A molecular technique (Conventional PCR with SG specific primers) was used to confirm that stored field isolate was *Salmonella* Gallinarum or not. The forward and revers primers and sequence used were SG-F 5'- GAT CTG CTG CCA GCT CAA- 3' and SG-R 5'- GCG CCC TTT TCA AAA CAT A -3' (Yang *et al*., 2014). Four replicate samples from the same hour grown culture on Brain heart infusion agar at log phase were used to amplify *glg*C gene and the control isolate was found from NVI molecular laboratory. The amplification reaction was carried out in a total volume of 20µl comprising 3µl nuclease free water, 2µl from each primer pair, 10µl IQTM super mix (BIO-RAD, USA) and 3µl template for one reaction. The PCR reaction conditions were 5 minutes at 94°C (initial denaturation step), 1 minute at 94° C (denaturation

step), 1 minute at 55° C (annealing step) and 1:30 minute at 72°C (extension step) for 35 cycles with final 10 minutes at 72° C (final elongation step) followed by holding at 4°C until the machine was off. Electrophoresis was run in TAE buffer by loading 10µl of the PCR product (mixed with loading dye) on 2% agarose gel wells stained with 2µl of ethidium bromide stock solution (10mg/ml). A parallel lane was also loaded with a 10µl molecular marker (ladder) and the products were separated at 120V for1hour then visualized under UV light and recorded (Adamu *et al*., 2017; NVI, 2016). PCR product with amplicon size of 174 bp was considered positive for *S*. Gallinarum (Kang *et al*., 2012; Adamu *et al*., 2017).

To determine the bacterial concentration (cfu/ml), 1ml of the suspension was withdrawn and serial dilutions ranging from 10^{-1} to 10^{-7} in 9ml of buffered peptone water was conducted. Then 0.1ml of suspension from each dilution was taken and poured on to the plate count agar and incubated for 24 hours at 37°C. Then the bacterial colonies were counted after 24 hours of incubation to determine time required to reach 10^{9} CFU/ml. Finally, the count was corresponded to the OD values read from the JENWAY 6305 UV/Vis spectrophotometer. At the same time optical density of bacterial suspension withdrawn at each time interval was measured and the relationship of OD with bacterial growth at time interval was determined.

3.3. Irradiation Dose Optimization for Field Bacterial Strain Inactivation

Irradiation was done using the Co60 gamma source (MDS NORDION, Canada and installed by India) at a National Institute for Control and Eradiation of Tsetse fly and Trypanosomosis (NICETT), Kality, Addis Ababa, Ethiopia.

After determination of CFU/ml, bacterial culture in brain heart infusion broth containing 5 and 10ml of falcon tubes in triplicates were centrifuged at 4000 rpm for 8 minutes at room temperature. The supernatant was discarded and pellets of cells were washed twice with PBS. It was then re-suspended in 5ml and 10ml PBS in triplicates of 15 ml falcon tubes and placed vertically at firm position in dry cell radiation chamber for inactivation. It was then exposed to different doses of gamma irradiation (1100, 1200, 1500, 1900, 2000, 2100, 2400, 2500, 2600, 2700, 2800, and 3000 grays of radiation) to attain complete inactivation and optimized doses of radiation to be used as

minimal lethal dose to 10^9 CFU/ml of organisms. The optimum irradiation dose for vaccine production was the lowest amount of radiation that was lethal to *Salmonella* Gallinarum (Abo-State *et al.,* 2010).

3.4. Investigation on Viability of Irradiated Bacteria

Viability of bacteria exposed to different irradiation doses was assessed by culturing on brain heart infusion agar (BHIA) and plate count agar to see if there is any growth. Samples were also enriched by Rappaport Vassiliadis *Salmonella* Enrichment Broth medium, to increase recovery of the bacteria. Suspected bacterial isolates were identified by biochemical tests such as indole, motility test, triple sugar iron agar, urea, citrate and lysine iron agar slants as described by (Lulie *et al.,* 2020).

3.5. Experimental Animals

A total of 60 female chickens (Sasso breed) at the age of 6 weeks were purchased from small scale poultry farm in Fitche town, located about 116 Km North of Addis Ababa. The farm receives and grows day-old chickens from a branch of Ethio-Chicken Private Limited Company. Chickens were kept in group of 5 chickens in cages with area of 1 meter by 1 meter and height of 90 cm in animal house of Aklilu lemma Institute of Pathobiology, Addis Ababa University as described by Lulie *et al*. (2020). Chicken's house at ALIPB was cleaned using detergents prior to the introduction of chickens and bedded with wood shavings (Sagatura). Chickens were kept in cages and allowed adlibitum access to antibiotics free fresh tap water and fed on balanced commercial feed prepared for layers, bought from Kality Animal Feed Processing Factory. Animals were allowed one-week of acclimatization following their arrival at the animal house of ALIPB and checked for serological status for antibody production against *S.* Gallinarium and *S.* Pullorum using slide agglutination test (SAT) (OIE, 2018). Chickens were vaccinated as against Marek's, Newcastle and Gumboro diseases as indicated on their record, while they were in multiplication farm they were taken from.

3.6. Study Design

The study design was a randomized experimental design. Animals were randomly assigned to six (6) groups having 10 chickens each were prepared for the experiment and each group had five chickens kept in sub-division cages found at Aklilu Lemma Institute of Pathobiology, Addis Ababa University. Different treatment groups were assigned randomly as, G_1 (vaccinated with irradiated *Salmonella* Gallinarum (SG) but unchallenged), G_2 (vaccinated with irradiated SG vaccine and then challenged), G_3 (vaccinated with irradiated SG vaccine twice at 21day interval (boostered) and challenged, G_4 (vaccinated with irradiated SG with 20% trehalose and challenged), G_5 (vaccinated with 9R commercial vaccine (Batch no 04-20) produced by NVI, Bishoftu, Ethiopia and challenged) and G_6 (unvaccinated but challenged). All chickens in their respective cages were coded and labeled-tags were tied on their leg firmly for identification of each bird to which group it belongs to**.**

3.7. Immunization and Challenge Experiment

Immunization was conducted using optimized gamma irradiated 10^9CFU/ml inoculums in 6 groups of Sasso breeds. Chickens arrived at ALIPB, just on the 6 weeks (42 days) of age and they were acclimatized for 1 week (up to day 49). All chickens were screened for antibody production against SG (using SAT) at the end of week 7 and all of them were negative for antibody of SG. The vaccination schedule was, first dose for G_3 started on week 8^{th} (day 56) of age and the booster for G_3 and first dose vaccination for G_1, G_2, G_4 and G_5 except G_6 (unvaccinated but challenged) was conduct on 11 weeks (day 77) of their age. Each group had 10 chickens and post exposure observation for clinical signs and gross pathological lesions on spleen, liver and gizzard was followed and recorded. All groups were vaccinated subcutaneously (S/C) at dose of 0.5 ml of 10^9 cfu/ml per chicken and records of level of safety and morbidity (depression, diarrhea, reluctant to move and anorexia) were taken every day. For the detection of SG antibody using the SAT test, blood samples were collected from wing vein at day 7, 14, 21 and 35 (booster) post vaccination and centrifuged at 12,000 rpm for 10 minutes to collect serum (Quinn *et al*., 1999). Level of antibody production was measured depending on the agglutination scores. Where, 1=weak, 2=moderate and 3 strong agglutination. Safety was measured by injection site reactions such as pain and

swelling, systemic reactions like fever and anorexia, and lesion in the liver, spleen and gizzard as described by Adamu *et al.* (2017).

The challenge for all groups was conducted 3 weeks post vaccination (14 weeks of age) using field isolates of *Salmonella* Gallinarum to both vaccinated and non-vaccinated groups except G_1 (vaccinated but not challenged group) through oral administration of 1 ml of bacterial suspension (PBS), that was in log phase of bacterial growth containing one field dose of *S.* Gallinarum ~ 5×10^7CFU/ml at the birds age of 8-16 weeks was given (OIE, 2018). Post challenge, birds were observed during which development of clinical signs and daily mortality was recorded. Post-mortem examination was conducted in all birds that were died during the challenge period and in all survivors at end of the challenge infection experiment, in which scoring of gross lesions were observed as described by Kiku *et al.* (2011) (Table 3).

Table 3: Experimental design for immunization and challenge infections

Groups	Types of vaccine	Immunization			Challenge infections			Samples taken		
		Route	Dose	No of chicken	Dose	No of chickens	Routes	Liver	Spleen	Gizzard
G_1	Irradiated	s/c	0.5	10	-	-	-	√	√	√
G_2	Irradiated	s/c	0.5	10	1ml	10	Oral	√	√	√
G_3*	Irradiated	s/c	0.5	10	1ml	10	Oral	√	√	√
G_4	Irradiated with 20%trehalose	s/c	0.5	10	1ml	10	Oral	√	√	√
G_5	Commercial 9R	s/c	0.2	10	1ml	10	Oral	√	√	√
G_6	Unvaccinated	-	-	10	1ml	10	Oral	√	√	√

Chickens in G_1-G_5: were immunized with single dose irradiated vaccine, G_3*: were vaccinated with booster dose on day 21of first vaccination, G_6: were unvaccinated with any of the vaccine type and except G_1, all groups G_2-G_6: were challenged

3.8. Evaluation of Protective Efficacy

The candidate irradiated SG vaccine was checked for its purity and safety as per the protocol stated by OIE (OIE, 2018). Its purity was evaluated by culturing the already

pelleted and PBS washed organism ready for radiation on to the Brain Heart Infusion Agar. Following vaccination, chickens were monitored daily for any unwanted reactions such as anorexia, depression, diarrhea, and death if any for 21 days. The liver, spleen and gizzard of dead chickens were examined for the presence of SG by plating on general and selective media.

At the end of the experiment, all chickens were sacrificed and a post-mortem examination was conducted to assess gross pathology in each group. Chickens were euthanized by cervical dislocation, which was carried out by grasping the leg of the chicken and stretching the neck and pulling on the head through applying a ventro-dorsal rotational force to the skull (Underwood *et al*., 2013). Gross lesions of enlarged and necrotic foci of the liver, spleen and gizzard were given scores of: 0, 1, 2, or 3. Where 0 stands for no lesion, 1 for mild necrotic foci on liver but not enlarged, 2 for enlarged liver but gizzard was not covered and 3 for more severe lesions which covered gizzard (Matsuda *et al*., 2011; Adamu *et al*., 2017; Lulie *et al*., 2020). Corresponding samples of liver, spleen and gizzard of dead chickens during the experiment and survived chickens at the end of experiment were taken aseptically during post-mortem examination for re-isolation of *Salmonella* Gallinarum. Samples of liver, spleen and gizzard corresponded to each bird was collected and cultured for isolation of *Salmonella* from birds that died during and at the end of the experiment. The protective efficacy of vaccine was calculated according to the formula developed previously (Orenstein *et al.*, 1985).

EV= (ARU-ARV)/ARU * 100 Where; EV= Efficacy of vaccine; ARU=Attack rate (morbidity and/or mortality) of unvaccinated group and ARV= Attack rate (morbidity and/or mortality) of vaccinated group (Orenstein *et al*., 1985).

3.9. Ethical Consideration

Ethical clearance was obtained prior to start of the experiment from the Institutional Review Board of Aklilu Lemma Institute of Pathobiology (ALIPB) (Ref.No: ALIPB/IRB/008/2015/16), Addis Ababa University.

3.10. Data Analysis

Graph Pad prizim (Version 9.0; GraphPad Software, Inc., La Jolla, CA, USA), was used to analyze data generated. Descriptive statistics was used to summarize clinical data (lesion score, immune responses, and bacterial re-isolation). Kruskal-Wallis was used to determine the significant difference between the groups of immunized (with radiation inactivated, 20% trehalose added and live attenuated commercial SG 9R vaccine groups) and unimmunized control groups. The significance of difference in survival of chickens in different treatment groups was determined using Log-rank (Mantel-Cox) survival curves. $P<0.05$ was considered as statistically significant.

4. RESULTS

4.1. Bacterial Strain Determination

Media selection test showed that turbidity and colony size on brain heart infusion broth and brain heart infusion agar respectively were found to be the best for the growth of test organism, which was followed by tryptone soya and nutrient medium (Table 4). Thus, brain heart infusion was used as culture media throughout the experiment.

Table 4: *Salmonella* Gallinarum growth condition in different media

Types of media used	Initial colony inoculum	Growth
Nutrient broth	3 identical SG	+
Brain heat infusion broth	3 identical SG	+++
Tryptone soya broth	3 identical SG	++
Nutrient agar	Loop full	+
Brain heat infusion agar	Loop full	+++
Tryptone soya agar	Loop full	++

Note: Less favourable (+); moderately favourable (++); very favourable (+++)

The electrophoresed PCR products on agarose gel revealed the expected size of the PCR products flanked by the primer pairs, which was positive for the *glg*C gene around 174 bp and similar to the positive control on lane P for *S*. Gallinarum (Figure 2).

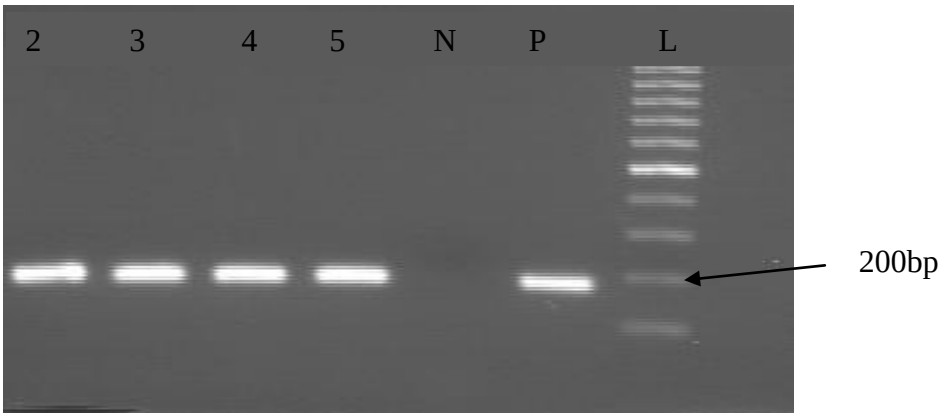

Figure 2: Gel image of the *Salmonella* Gallinarum serotype specific PCR reaction

Lane L: 100bp Molecular marker or ladder; Lane 2-5: Duplicate samples of *Salmonella* Gallinarum; Lane N: Negative control and Lane P: Positive control stored at NVI molecular laboratory.

4.2. Bacterial Growth Dynamics

Salmonella Gallinarum (six similar colony) taken after 18 hrs of growth on brain heart infusion agar (Oxoid, UK) and transferred to brain heart infusion broth and observed for consecutive six hours growth showed that CFU/ml versus time (t) was an exponential growth (Figure 3a). The optical density of SG versus time (t) graph showed increment while it was in log phase and kept constant when it approached the stationary phase. The optical density (OD) doesn't measure viable cells count decrement at stationery and death phase (Figure 3b).

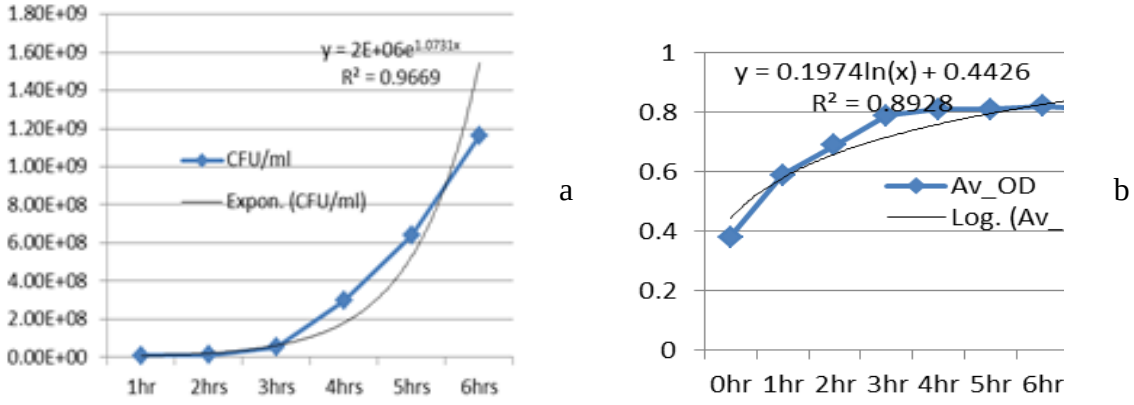

Figure 3: Graphs showing (a) colony forming unit per ml and (b) optical density values versus time

4.3. Radiation Dose Optimization and *Salmonella* Gallinarum Viability

Salmonella Gallinarum exposed to doses greater than or equal to 2600 Gy of irradiation was completely inactivated and growth was not observed. However, the candidate vaccine with 20% trehalose irradiated with 2600 Gy showed limited growth and viability of SG disappeared at dose of greater and equal to 2800Gy. Its growth was also observed by culturing on BHIA and RV for 24-48hrs. Therefore, irradiation dose of

2600Gy was selected to inactivate and observe the immunogenicity and protective efficacy emanated from the differences (Table 5).

Table 5: Growth status of *Salmonella* Gallinarum exposed to different doses of gamma radiation

Bacterial concentration (OD at 600nm)	Irradiation Dose (Gy)	Growth on BHIA (24hrs)	Growth after enriching on RV(48hrs)
0.8	3000	-ve	-ve
0.8	2800	-ve	-ve
0.8	2700	-ve	-ve
0.8	2600	-ve	-ve
0.8	2500	-ve	+ve
0.8	2400	+ve	+ve
0.8	2100	+ve	+ve
0.8	2000	+ve	+ve
0.8	1900	+ve	+ve
0.8	1500	+ve	+ve
0.8	1200	+ve	+ve
0.8	1100	+ve	+ve

OD= Optical Density measured at 600 nm, -ve= No bacterial growth, +ve= There was bacterial growth, BHIA=Brain heart infusion agar, RV= Rappaport Vassiliadis

4.4. Safety and Immunogenicity of Irradiated Candidate Vaccine

As there was no growth of organisms observed after inoculation into the media at 37°C for 24-48 hours, the irradiated vaccine was considered biologically pure. For the safety concern, except group of chickens exposed to the 20% trehalose added (as radiation protectant) and irradiated vaccine group (G_4) which showed depression and loss of appetite from day 3-7 and recovered soon, all vaccinated chickens showed no clinical signs which indicated that irradiated vaccine at irradiation dose of 2600 Gy was safe.

However, all birds tested at day 7 and onwards were found sero-positive post vaccination (Table 6).

Eight (80%) of chickens immunized with irradiated SG vaccine (G_1) showed weak reaction to antigen whereas 2(20%) showed moderate reaction on day 7 post vaccination. However, on day 14, all of these chickens showed moderate reaction and on day 21, 5(50%) of them showed strong reaction. Three (30%), 7(70%) of chickens exposed to irradiated vaccine in group two (G_2) showed weak and moderate reaction respectively on the 7^{th} day whereas 2(20%) and 8(80%) G_2 showed weak and moderate reaction on day 14 respectively. However only 2(20%) of G_2 showed strong reaction and 8(80%) remained on moderate reaction to the antigen on day 21 post vaccination. Group three (G_3) showed weak 4(40%) and moderate 6(60%) reaction on 7^{th} day post vaccination, whereas 2(20%) and 8(80%) of them showed weak and moderate reaction on day 14 respectively. On day 21, 7(70%) and day 35(booster dose), 4(40%) of G_3 showed moderate reaction. Two (20%) and 8(80%) of G_4 showed weak and moderate reaction on day 7 respectively. However, 5(50%) and 2(20%) of G_4 showed moderate reaction on days 14 and 21 respectively. Group five (G_5) showed 3(30%) weak and 7(70%) moderate reaction on day 7. Four (40%) moderate and 6(60%) strong reactions of G5 were recorded on day 14. However, 1(10%) moderate and 9(90%) strong reactions were seen on day 21 of post vaccination in the same group (G_5). Only 20% and 30% of G_2 and G_3 respectively produced strong antibody reaction on day 21 of first dose of vaccination. However, group four (G_4) and five (G_5) showed strong antibody production than other groups on day 21 (80, 90%) respectively. But G_3 on booster dose after 2 weeks of vaccination showed 60% of strong agglutination reaction (day 35 from first day of vaccination). The negative control group (G_6) showed no reaction on days 7, 14, 21 and 35. Except G_3 (on booster dose), G_4 and G_5 which showed strong reaction from day 14 and onwards, other groups (G_1, G_2 and G_3) on single dose of irradiated vaccine showed strong reaction on day 21 of post vaccination (Table 6).

Table 6: Sero-positivity of serum samples taken from different groups post exposure to 0.5 ml of irradiated vaccine at 10^9 cfu/ml and 0.2 ml conventional 9R strain vaccine at 10^7cfu/ml subcutaneously

Group (n-10)	Types of vaccine	Days after vaccination	Sero-positive samples (%)		
			Weak	Moderate	Strong
Group-1	Irradiated	7	8 (80)	2(20)	0(0)
		14	0(0)	10(100)	0(0)
		21	0(0)	5(50)	5(50)
Group-2	Irradiated	7	3(30)	7(70)	0(0)
		14	2(20)	8(80)	0(0)
		21	0(0)	8(80)	2(20)
Group-3	Irradiated	7	4(40)	6(60)	0(0)
	Booster	14	2(20)	8(80)	0(0)
		21	0(0)	7(70)	3(30)
		35	0(0)	4(40)	6(60)
Group-4	Irradiated with 20%	7	2(20)	8(80)	0(0)
	trehalose	14	0(0)	5(50)	5(50)
		21	0(0)	2(20)	8(80)
Group-5	9R commercial	7	3(30)	7(70)	0(0)
	Vaccine	14	0(0)	4(40)	6(60)
		21	0(0)	1(10)	9(90)
Group-6	Negative control	7	0(0)	0(0)	0(0)
		14	0(0)	0(0)	0(0)
		21	0(0)	0(0)	0(0)
		35	0(0)	0(0)	0(0)

Note: SC= Subcutaneous route; n=stands for the number of chickens per each group

4.5. Protective Efficacy of Irradiated *Salmonella* Gallinarum Vaccine

Log-rank (Mantel-Cox) for comparison of survival curves showed that there was significant difference between the survivors of vaccinated and non-vaccinated group ($P<0.0001$). There was significant difference ($p<0.05$) in level of protection between G_2 and G_4 as well as between G_2 and G_5. Survivors in G_1 and G_5 were 100% that showed commercial 9R vaccine conferred strong protection as compared to G_2 (vaccinated and challenged), G_3 (booster vaccinated and challenged), G_4 (vaccinated with 20% trehalose added vaccine and challenged) and G_6 (unvaccinated but challenged) and their protection for G_2, G_3 and G_4 was 20%,70% and 70% respectively. Hence G_6 was unvaccinated; protection from irradiated SG vaccine was not expected. The survival comparisons curve showed that there was no statistically significant protection in chickens in G_2 when compared to those in G_6 ($P>0.05$) and indicated that the

completely inactivated candidate vaccine at radiation dose of 2600Gy was less immunogenic hence the protection level was equal to the unvaccinated group and naturally 20% survived chickens. Addition of 20% trehalose sugar and booster dose improved its protection level by 50% (Figure 4). Death events were recorded from 6 to 13 days which indicates 15 days of follow up from first day of challenge seems enough for such experiment (Figure 4).

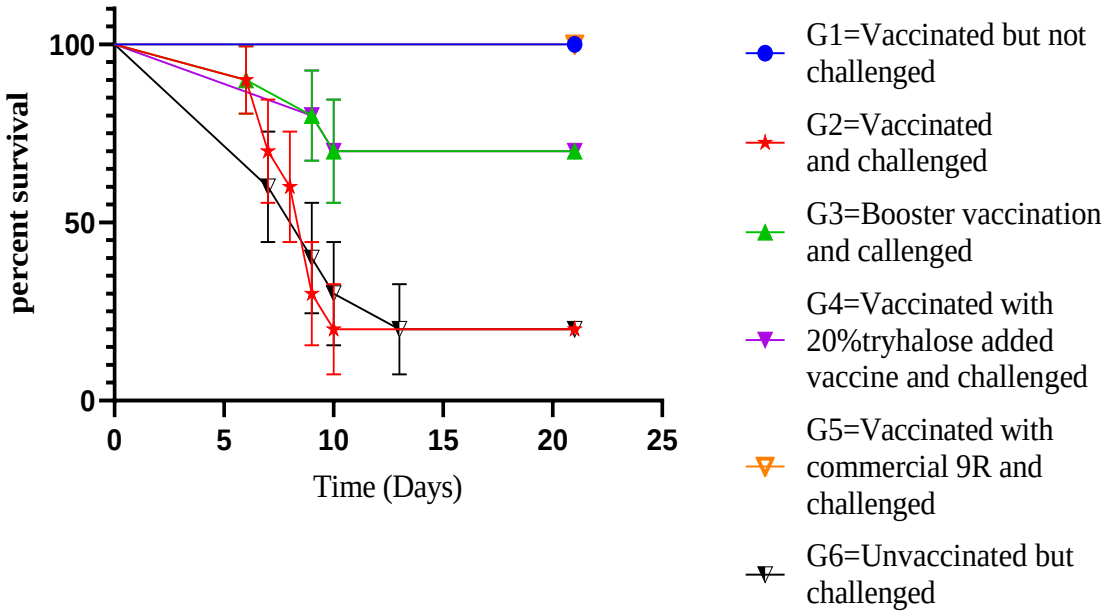

Figure 4: Protections conferred in chickens vaccinated with whole cell gamma-irradiated *Salmonella* Gallinarum candidate vaccine formulations upon challenge experiment

4.6. Follow up, Gross Lesion and Bacterial Recovery

Before challenge, chickens in G_4 (irradiated and 20% trehalose added vaccine) group showed signs of depression and loss of appetite for not more than five days and recovered soon after day seven. However, other groups vaccinated with irradiated and 9R commercial vaccine and negative control groups were found healthy throughout the immunization period. During the challenge period, except the unchallenged group (G_1) and the commercial 9R live attenuated vaccine group (G_5), all chickens belonging to different groups manifested mild to severe clinical signs of depression, diarrhea, loss of appetite and death.

As severity developed, all chickens in challenged groups died within 8 days from the first day of death record (day 6). The total death events following challenge were; at day 6 (2), day 7 (6), day 8 (1), day 9 (8), day 10 (4) and day 13 (1). The highest overall death per day was 8(36.36%) of the total death which was recorded on 9^{th} day of challenge. Chickens survived and died were investigated for the presence and absence of *Salmonella* Gallinarum. All corresponding samples of liver, spleen and gizzard taken from survived chickens after challenge were found negative for *S*. Gallinarum on day 33 of challenge infection. However, 22(100%) liver, 22(100%) spleen and 18 (81.81%) gizzard samples taken from died chickens during challenge period were positive for the bacterial recovery test. Gross lesions from dead and survived chickens were observed and recorded (Table 7). There was statistically significant difference ($p<0.05$) between the lesion scores found in died and survived flocks during and at the end of the experiment, respectively. Of the total death (22) during challenge, 21 (95.45%) of them were found with mild lesion on liver. However, 78.95 % of survived and sacrificed chickens at the end of the challenge experiment were found with no lesion on liver, spleen and gizzard (Table 7).

Table 7: Mortality during the experiment, survivors at the end of experiment, lesions and bacterial recovery during and at the end of challenge

Group (n=10)	No died	No survived	Lesions in died (%)	Lesions in survived (%)	Efficacy (%)	Bacterial recovery (%)	P-Value
G-1	0	10	0 (0)	0 (0)	-	0(0)	
G-2	8	2	8 (100)	0 (0)	0	8(100)	
G-3	3	7	2 (66.66)	3 (42.85)	62.5	3(100)	
G-4	3	7	3 (100)	2 (28.57)	62.5	3(100)	
G-5	0	10	0 (0)	3 (30)	100	0(0)	
G-6	8	2	8 (100)	0 (0)	-	8(100)	
Total	22	38	21(95.45)	8 (21.05)	-	22(100)	P<0.05

G_1=vaccinated with irradiated vaccine but not challenged; G_2=vaccinated with irradiated vaccine and challenged; G_3=vaccinated twice at 21 days interval (booster vaccinated) and challenged; G_4=vaccinated with irradiated 20% trehalose added vaccine and challenged; G_5=vaccinated with commercial 9R vaccine produced by National Veterinary Institute, Ethiopia (NVI); G_6=unvaccinated but challenged group.

5. DISCUSSION

Fowl typhoid is caused by *Salmonella enterica* subspecies *enterica* serovar Gallinarum biovar Gallinarum (Vaid *et al*, 2015). It is poultry disease causing septicaemia, morbidity, mortality and inflammation in organs like liver and spleen which results significant economic losses to commercial/back yard poultry farms globally (Shivaprasad, 2000; Barrow and Neto, 2011). Vaccination is the most important practice and strategy used to control fowl typhoid.

In recent vaccine development, radiation inactivation has been used to develop a variety of vaccine types, because of its ability to eradicate chemical contaminants and penetrate pathogens to destroy nucleic acids without damaging the pathogen surface antigens and radiation inactivated vaccines showed better protection efficacy than the conventional vaccines but the type of response induced may differ depending on the type of pathogen (Seo, 2015; David *et al*., 2017; Ji *et al*., 2021).

In current study, the test showed typical characteristics of *Salmonella* and it was consistent with previous studies (Mdegela *et al*., 2000; OIE, 2014). Molecular identification of the isolates based on serotype specific PCR assay revealed that the fidelity and discriminatory power of the conventional method used to detect *Salmonella* Gallinarum was rapid and definitive (Shah *et al*., 2005; Kang *et al*., 2012; NVI, 2016; Adamu *et al*., 2017).

The bacterial dose optimization, the colony forming unit/ml related to its optical density used in this study was in agreement with Ji *et al*. (2021), which OD=0.8 was approximately equivalent to 10^9cfu/ml.

All radiation doses greater than 2.6 kGy were found lethal to *S.* Gallinarum in the current study, which agrees with previous work that reported) no bacterial growth after irradiating 10^8-10^9 CFU/ml of *S.* Gallinarum at 3-4 KGy in (Ji *et al.*, 2021). However, the minimum lethal dose found to inactivate the inoculum was 2.6 kGy, which was in agreement with the study by Lule *et al.* (2020). Minimum lethal dose should be used not to damage the epitopes required to develop a protective immune response while

maintaining an adequate margin of safety to ensure complete inactivation (Dollery *et al*., 2021). However, adding 20% tryhalose sugar protected the SG from being damaged by radiation as Yoshinaga *et al.* (1997) suggested that trehalose protected DNA from being damaged by gamma radiation. Therefore, radiation dose and sugar proportion optimization for trehalose added SG vaccine is important to prepare higher protective efficacy of irradiated vaccine for fowl typhoid. Gamma radiation was chosen in this experiment over other means of radiation for SG inactivation because of its highly penetrative property, insignificant residual effect (safety), less exposure time required (FDA, 2000).

Inactivated (killed) *Salmonella* vaccines are less effective in clearing the disease-causing organisms from the internal organs as compared to the live vaccines (Gast *et al*., 1993). This might be because of humoral immunity alone is unlikely to fully protect (de Paiva *et al*., 2009). Total protection needs both humoral and cellular immunity (Miyamoto *et al*., 1999). Recent novel adjuvant technologies are promising in development of inactivated and safe *Salmonella* vaccines which induce stimulation to different target immune system of chickens (Barrow, 2007). Even though further optimization is required, addition of 20% tryhalose sugar (as radiation protectant) in candidate SG vaccine in current study also showed promising result as compared to the vaccines formulated as totally killed and free of trehalose sugar, which was in agreement with Yoshinaga *et al*. (1997) that reported trehalose protected the genetic material of bacteria and increased the immunogenicity of the vaccine.

Vaccine administration in current experiment was through subcutaneous route as suggested by Lulie *et al*. (2020) that subcutaneously administered SG vaccine against fowl typhoid showed better intensity and level of antibody production than oral route. In this study, almost all chickens vaccinated with SG vaccine showed weak sero-positivity starting from day 7 and improved at day 14 and 21 to moderate and strong positivity depending on the type of vaccine and treatment groups. This indicates that killed vaccine was poor in eliciting antibody production as compared to the live attenuated commercial 9R and 20% trehalose added (inactivated but not totally killed) vaccines, which was in agreement with the studies (Miyamoto *et al*., 1999; Lulie *et al*., 2020; Dollery *et al*., 2021). Previous study showed that radiated SG vaccine provided a higher homogeneous protective response against group D *Salmonella* by activating

early humoral responses (IgG2b and IgG3) and Th1/Th17 cell-mediated immunity than formalin inactivated SG vaccine (Ji *et al*., 2021).

Despite the study by Lulie *et al*. (2020) which revealed that SG vaccine irradiated with 2.4 kGy and 9R commercial vaccine protected equally (50%) of the chicken (Bovan brown chickens) on challenge, the booster dose of SG vaccine irradiated with 2.6 kGy and first dose of 20% trehalose added vaccine protected 70% of the flock (brown egg layer Sasso breed chickens) on challenge experiment in the current study. Even though the 2.6 kGy irradiated SG vaccine revealed less immunogenicity as compared to 2.4 kGy irradiated vaccine (Lulie *et al*., 2020), breed difference and the booster dose of the vaccine might be the reason for the improvement of protective efficacy in this study. However, the commercial 9R vaccine in current study protected 100% of the chickens, which was in agreement with Adamu *et al*. (2017). In contrary to the studies by Adamu *et al*. (2017) and Lulie *et al*. (2020), which revealed 53.4% and 100% mortality in unvaccinated chickens respectively, the current study showed 80% mortality in both totally killed single dose SG vaccine in G_2 and unvaccinated groups (G_6). As stated by Kwon and Cho (2011), the level of protection efficacy could vary due to the difference in breed type, susuptibility, vaccinal inoculum and bacterial challenge doses.

The present study also revealed that vaccinated and survived chickens from the challenge infection experiment showed significantly reduced number of gross lesions on organs (liver and spleen) and none of the chickens vaccinated and survived at the end of the challenge experiment was found positive for *S.* Gallinarum on bacterial re-isolation, which was almost in line with the study by Adamu *et al*. (2017) and Lulie *et al*. (2020).

6. CONCLUSION AND RECOMMENDATIONS

In the current study, booster dose of 2600 Gray irradiated (killed) SG vaccine and addition of trehalose (20%) on SG during irradiation as protectant revealed promising results, which both formulations showed significant protection (70%) in the homologous challenge infection experiment. In this study, the live attenuated commercial 9R vaccine was found with best efficacy, which protected 100% of the flock against the fowl typhoid caused by the field strain of *Salmonella* Gallinarum.

Considering the conclusion drawn above, the following recommendations are forwarded to improve protective efficacy of the SG candidate vaccine;

- The effect of booster dose on the protective efficacy of the candidate vaccine should be evaluated under different management condition using larger sample size of different breeds of chicken
- Optimization of radiation dose for inactivation of SG (with the non-motile and easily sedmenting property) and the amount of trehalose sugar to be used in the vaccine formulation should be conducted
- In addition, investigation works on improving the immunogenicity of the vaccine using radio-protective agents such as Mn^{2+} peptide complex should be in place
- Quantification of antibody response using ELISA and other immunological methods.
- Further studies should be conducted on the stability, periods of immune response and protection, and shelf life of the gamma irradiated SG candidate vaccine

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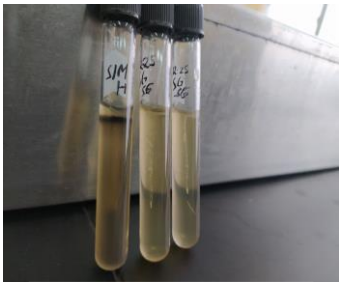
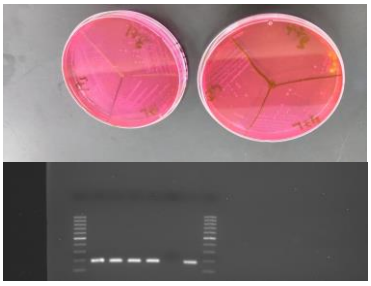
8. ANNEXES

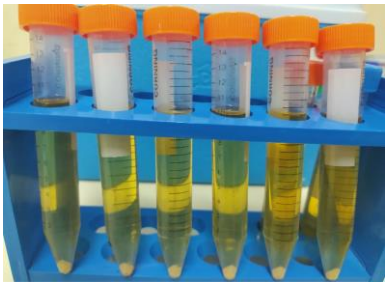
Annex 1: Photo gallery



SIM test



Strain determination (PCR)



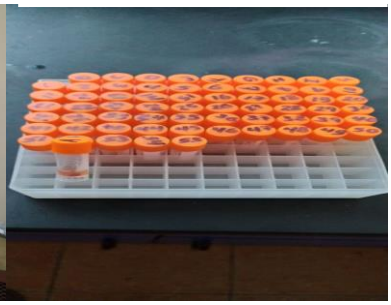
Pellets in BHIB

Washed pellets in PBS

Co60 radiation source

Chickens in the cage

Blood collection

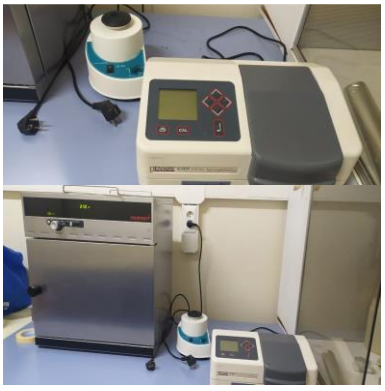


Corresponding Serum in Vials

Serial dilution in ALIPB Lab

Challenge infection

Sacrificed birds + samples

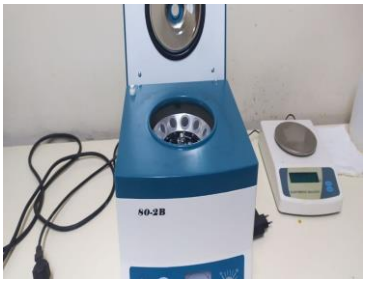


Spectrometer& incubator

Cages for chickens

-ve plates after radiation

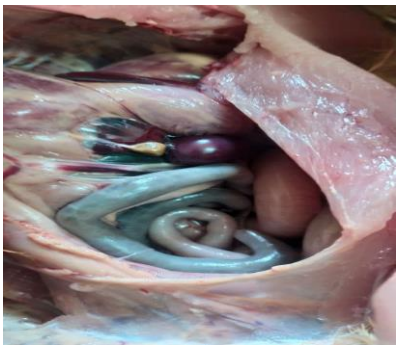
PM- examination



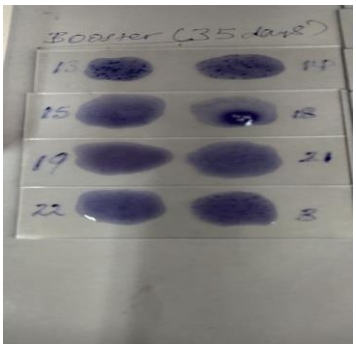
Test tube centrifuge

Enlarged spleen

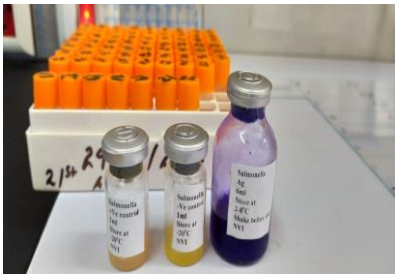
Enlarged (L) & normal(R) liver



Dislocation

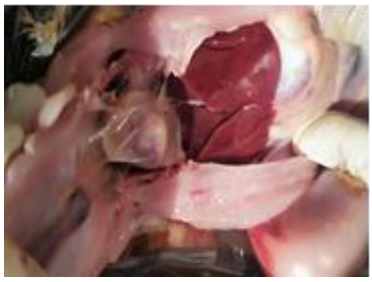


Slide agglutination test (SAT)

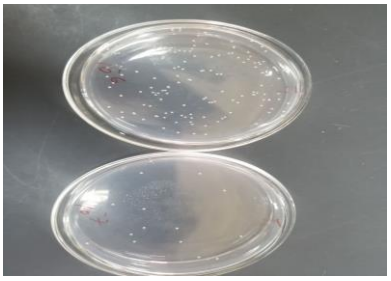


-ve, +ve controls and Antigen

9R commercial vaccine



Liver with necrotic foci



Countable colonies (<300)

Colony counter

Annex 2: Serial dilution to determine colony forming unit per ml using bacteria plate count method

Plate codes	Dilut ions	Dilution factors	ml of dilution plated	Final dilution on the plate	Number of colonies	Bacterial count pre ml of sample /CFU/ml	Duration of growth
1	10^{-1}	10^{1}	0.1ml	1:10	TNTC		
2	10^{-2}	10^{2}	0.1ml	1:100	TNTC		
3	10^{-3}	10^{3}	0.1ml	1:1000	TNTC		
4	10^{-4}	10^{4}	0.1ml	1:10000	93	9.3×10^{6}	1hr
5	10^{-2}	10^{2}	0.1ml	1:100	TNTC		
6	10^{-3}	10^{3}	0.1ml	1:1000	TNTC		
7	10^{-4}	10^{4}	0.1ml	1:10000	129	1.29×10^{7}	2hrs
8	10^{-5}	10^{5}	0.1ml	1:100000	TFTC(20)		
9	10^{-3}	10^{3}	0.1ml	1:1000	TNTC		
10	10^{-4}	10^{4}	0.1ml	1:10000	TNTC		
11	10^{-5}	10^{5}	0.1ml	1:100000	53	**5.3×10^{7} ***	3hrs
12	10^{-6}	10^{6}	0.1ml	1:1000000	TFTC(10)		
13	10^{-4}	10^{4}	0.1ml	1:10000	TNTC		
14	10^{-5}	10^{5}	0.1ml	1:100000	295	2.95×10^{8}	4hrs
15	10^{-6}	10^{6}	0.1ml	1:1000000	TFTC(25)		
16	10^{-7}	10^{7}	0.1ml	1:10000000	TFTC(6)		
17	10^{-4}	10^{4}	0.1ml	1:10000	TNTC		
18	10^{-5}	10^{5}	0.1ml	1:100000	TNTC		
19	10^{-6}	10^{6}	0.1ml	1:1000000	64	6.4×10^{8}	5hrs
20	10^{-7}	10^{7}	0.1ml	1:10000000	TFTC(4)		
21	10^{-8}	10^{8}	0.1ml	1:100000000	0		
22	10^{-1}	10^{1}	0.1ml	1:10	TNTC		
23	10^{-4}	10^{4}	0.1ml	1:10000	TNTC		
24	10^{-5}	10^{5}	0.1ml	1:100000	TNTC		
25	10^{-6}	10^{6}	0.1ml	1:1000000	116	**1.16×10^{9} ****	6hrs
26	10^{-7}	10^{7}	0.1ml	1:10000000	TFTC		
27	10^{-8}	10^{8}	0.1ml	1:100000000	TFTC		

* Inoculum used for challenge
** Used as candidate vaccine
Bacterial count > 300 colony was considered as too numerous to count (TNTC)
Colony counts < 30 were also considered as too few to count (TFTC)

Annex 3: Slide agglutination test protocol

Test procedure

- Before starting the test, bring all reagents at room temperature
- Thaw test serum, positive and negative serum(PBS) at room temperature
- Place 30 µl of known antigen on the clean slide
- Place 30 µl of tested serum near to the antigen
- Mix well the antigen and serum using micropipette tips
- Gently shake for 4 minutes
- Interpretation: - Agglutination indicate positive result
  - No agglutination indicates negative result

Annex 4: Media compositions and preparation procedures

I. Buffered peptone water (BPW)

Composition

Ingredient	gm/liter
Disodium Phosphate	3.2
Monopotassium Phosphate	1.5
Sodium Chloride	5
Peptone	10
Final PH: 7.2+/- 0.2 at 25°C	

Direction:

- Suspend 20.0gm in 1 liter distilled water
- Heat gently if necessary to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes

II. Lysine Iron Agar (LIA)

Composition

Ingredient	gm/liter
Peptone	5.0
Yeast extract	3.0
Glucose	1.0
L-lysine	10.0
Ferric ammonium citrate	0.5
Sodium thiosulphate	0.04
Bromo ceresol purple	0.02
Agar	15

Final PH: 6.7±0.2 at 25°C

Direction:

- Suspend 34.56 gm in 1-liter distilled water
- Heat to boiling to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes
- Cool the tubes in slanted position to form slants with deep butts

III. Nutrient Agar (NA)

Composition

Ingredient	gm/liter
Peptone	5.0
Sodium Chloride	5.0
Beef extract	3.0
Agar	15
Final PH: 7.4± 0.2 at 25°C	

Direction:

- Suspend 28.0 gm in 1 liter distilled water
- Heat to boiling while stirring to dissolve the medium completely
- Sterilized by autoclaving at 115°C for 15 minutes
- Pour in to each sterile petri plate on the sterile surface until the agar has solidified

IV. Nutrient Broth (NB)

Composition

Ingredient	gm/liter
Peptone	5.0
Sodium Chloride	5.0
Beef extract	3.0
Final PH: 7.4± 0.2 at 25°C	

Direction:

- Suspend 34.56 gm in 1 liter distilled water
- Heat to boiling to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes

V. Plate Count Agar (Tryptone Glucose Yeast Agar)= PCA

Composition

Ingredient	gm/liter
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar	9
Final PH: 7.0± 0.2 at 25°C	

Direction:

- Suspend 17.5 gm in 1 liter distilled water
- Heat to boiling while stirring to dissolve the medium completely
- Sterilized by autoclaving at 115°C for 15 minutes
- Pour in to each sterile petri plate on the sterile surface until the agar has solidified

VI. Rapport Vasiliadis Broth (RV)

Composition

Ingredient	gm/liter
Soya Peptone	5
Sodium Chiloride	8.0
Monopotassium Phosphate	1.6
Magnesium Chloride, anyhydrous	18.7
Malachite green	0.04
Final PH: 6.9±0.2 at 25°C	

Direction:

- Suspend 30.0 gm in 1 liter distilled water
- Heat to boiling to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes

VII. Simmons Citrate Agar

Composition

Ingredient	gm/liter
Sodium Chiloride	5.0
Sodium Citrate	2.0
Ammonium Dihydrogen Phosphate	1.0
Dipotassium Phosphate	1.0
Magnesium Sulphate	0.2
Bromothymol Blue	0.08
Agar	15
Final PH: 6.9±0.2 at 25°C	

Direction:

- Suspend 24.28 gm in 1 liter distilled water
- Heat to boiling to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes
- Cool the tubes in slanted position to form slants with deep butts

VIII. SIM (Sulfide, Indole, Motility) medium

Composition

Ingredient	gm/liter
Pancreatic digest casein	20.0
Peptic digest of animal tissue	6.1
Ferrous Ammonium Sulfate	0.2
Sodium Thiosulfate	0.2
Agar	3.5
Final PH: 7.3±0.2 at 25°C	

Direction:

- Suspend 30.0 gm in 1 liter distilled water
- Heat to boiling to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes

IX. Triple Sugar Iron Agar (TSI)

Composition	
Ingredient	gm/liter
Beef extract	3.0
Yeast extract	3.0
Pancreatic digest casein	15.0
Proteone Peptone	5.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous sulfate	0.2
Sodium thiosulphate	0.3
Sodium Chloride	5.0
Phenol red	0.024
Agar	12
Final PH: 6.7±0.2 at 25°C	

Direction:

- Suspend 64.52 gm in 1 liter distilled water
- Heat to boiling to dissolve the medium completely
- Dispense as desired into the tube and sterilized by autoclaving at 115°C for 15 minutes
- Cool the tubes in slanted position to form slants with deep butts

X. Urea Agar Base

Composition	
Ingredient	gm/liter
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Disodium Phosphate	1.2
Monopotassium Phosphate	0.8
Phenol red	0.012
Agar	15
Final PH: 6.8±0.2 at 25°C	

Direction:

- Suspend 24.01 gm in 950ml distilled water
- Heat to boiling to dissolve the medium completely
- Sterilized by autoclaving at 115°C for 15 minutes
- Cool to 45-50°C and aseptically add 50ml of sterile 40% urea solution and mix well

- Dispense into sterile tubes and allow to set in a slant position to form slants with deep butts

XI. Xylose-Lysine-Deoxycholate Agar (XLD)

Composition

Ingredient	gm/liter
Lactose	7.5
Sucrose	7.5
Sodium Thiosulfate	6.8
L-lysine	5.0
Sodium Chloride	5.0
Xylose	3.75
Yeast extract	3.0
Sodium Deoxycholate	2.5
Ferric Ammonium citrate	0.8
Phenol red	0.08
Agar	15
Final PH: 7.2±0.2 at 25°C	

Direction:

- Suspend 56.68 gm in 1 liter distilled water
- Heat to boiling while stirring to dissolve the medium completely
- Avoid overheating and do not autoclave
- Transfer immediately to a water bath at 50°C
- Then pour in to each sterile petri plate on the sterile surface until the agar has solidified

Annex 5: Letter of ethical clearance

Aklilu Lemma Institute of Pathobiology
Addis Ababa University
Institutional Review Board

Approval Sheet

Minutes Ref No.: ALIPB/IRB/008/2015/16

Date: Monday September 26, 2016

Title of the Research Proposal: **"Development of irradiated vaccine for control of fowl typhoid in poultry"**

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PI: Tadesse Eguale (PhD)

Recommendation of the ALIPB Institutional Review Board

The ALIPB Institutional Review Board has noted the input of the above mentioned research proposal to the poultry production of the country. The proposal has been approved by the ALIPB/IRB dated September 26, 2016. The PI should submit progress report for the work every 6 months and the final report upon completion. The PI should also notify the ALIPB/IRB ahead of time any amendments or modifications in the protocol or premature suspension or termination of the study.

STATUS: APPROVED WITH RECOMMENDATION

Chairperson
Name: Dr. Tilahun Teklehaymanot

Signature:_____

Secretary
Name: Dr. Lemu Golassa

Signature:

Approval
Name: Dr Mirutse Giday, Director

Signature:

Date:

ADDIS ABABA UNIVERSITY