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The Oral Application of the Onderstepoort Biological Products Fowl Typhoid Vaccine, its Safety, Efficacy and Duration of Protection in Commercial Laying Hens

By

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ABSTRACT

This project was undertaken to establish whether the Onderstepoort Biological Products Fowl Typhoid (OBPft) vaccine registered as an injectable vaccine was effective and safe when administered orally to commercial layers. Its efficacy and duration of protection were compared to the intramuscular injectable route. Commercial brown layer hens were used as they were found to be highly susceptible to *Salmonella gallinarum* infections.

In the safety trial birds were euthanased at timed intervals spanning 4-weeks post vaccination. Necropsies were performed and samples were taken and tested. No clinical signs or mortalities could be attributed to the OBPft vaccine. No active shedding of the vaccine strain could be detected. Slight pathological changes were noted with both routes of vaccination; however these changes were transient, returning to normal within the observation period. The injected group showed a better serological response with the serum agglutination test than the orally vaccinated groups.

In the duration of protection trial the two routes of vaccination were compared, the birds were challenged at three 8-week intervals post vaccination. All the unvaccinated birds died. The protection offered to the vaccinated groups was good when birds were challenged 8 and 16-weeks after vaccination. However, this dipped steeply by the challenge 24-weeks post vaccination. Statistically (ANOVA, $p<0.05$) it was found that there was no significant difference between the protection offered by either the oral or injected route of vaccination with the OBPft vaccine.

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ABBREVIATIONS

ATCC	American type culture collection
BHI	Brain heart infusion
BCA	Blood Columbia Agar
BPW	Buffered peptone water
BWD	Bacillary White Diarrhoea
cfu	Colony forming units
CI	Confidence Interval
FT	Fowl typhoid
GALT	Gut associated lymphoid tissue
H_A	Alternate Hypothesis
H_O	Null hypothesis
Ig	Immunoglobulin
LPS	Lipopolysaccharide
MAC	MacConkey agar
M cell	Microfold cell
MDT	Mean Death Time

MS	Mean Square
neg	Negative
No.	Number
OBP	Onderstepoort Biological Products
OBPft	Onderstepoort Biological Products fowl typhoid vaccine
ODI	Optical Density Index
PCR	Polymerase Chain Reaction
PM	Post mortem examination
pos	Positive
PRC	Poultry Reference Centre
SE	*Salmonella enterica* serovar Enteriditis
SG	*Salmonella enterica* biotype *gallinarum*
SG9R	Salmonella Gallinarum rough vaccine strain
SP	*Salmonella enterica* biotype *pullorum*
SS	Sum of Squares
vac	Vaccination
wt	Weight
XLD	Xylose lysine decarboxylase

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CHAPTER 1

1.1 INTRODUCTION

As poultry and their products are an important source of protein for South Africa and many other third-world economies, infections due to *Salmonella* can have a major impact on the profitability of this industry. Although many of the countries in Europe and North America have reduced the prevalence of *Salmonella* infections, in Africa, including South Africa, Asia, South America and the Middle East the number of reported outbreaks of especially fowl typhoid are increasing (Saif, 2003; Silva, Snoeyenbos, Weinack and Smyser, 1981a).

Salmonella may be divided into three categories on the basis of the diseases caused, their host species range and invasiveness. The first group includes the highly pathogenic chicken-adapted *S. enterica* biotype *gallinarum* (SG) and *S. enterica* biotype *pullorum* (SP), which cause fowl typhoid and pullorum disease respectively. The second group includes the invasive serotypes of *Salmonella*, namely *S. enterica* serotype Enteritidis and *S. enterica* serotype Typhimurium that can cause serious disease in humans. The third group are the non-invasive *Salmonella*, which rarely causes illness in birds or humans. However, more outbreaks of unusual serotypes are being experienced in humans (Saif, 2003).

This trial focused on the immunoprophylaxis of fowl typhoid, a disease that causes marked losses in areas where it occurs (Saif, 2003).

Since fowl typhoid has largely been eradicated from the developed parts of the world there has been little need for research in this area in those countries since 1960. By 1990 it became clear that the situation was very different in developing countries where the disease prevalence was increasing and measures had to be taken to better understand the

pathogen, the host-parasite interaction and re-evaluate older control methods, as well as finding new and improved ways to control this disease.

There are several ways in which the disease can be eradicated or controlled. Eradication programmes used in Europe have proven to be too costly for developing countries. They were based on the regular serological testing of sentinel birds and euthanasia of positive flocks. These farms were then thoroughly disinfected and restocked with disease-free birds. They then ensured a high level of biosecurity and continued to serologically monitor for the disease. An alternative, more attractive, option to developing countries like South Africa was to control fowl typhoid by immunoprophylaxis and good farm hygiene.

At present two live fowl typhoid vaccines are registered for use in South Africa: Nobilis SG9R (Intervet, Netherlands), containing a live attenuated rough strain of SG (SG9R) and the Onderstepoort Biological Products Fowl Typhoid (OBPft) live vaccine which contains the 5503 rough attenuated strain of SG (Cameron and Buys, 1979). Both are administered via the subcutaneous route. However, this route poses a problem as the vaccinators must be specifically trained in this procedure, this application is time consuming, some hens may not be vaccinated if large numbers are being immunised and the vaccine once mixed must be used immediately.

Outbreaks of SG in layers still occur in South Africa despite extensive vaccination. In a number of SG9R vaccine trials a higher percentage of unvaccinated birds died (15–35%) from fowl typhoid than vaccinated birds (6–15%) (Harbourne, 1963). Thus vaccination decreases mortality in the birds but not the presence of disease. Although there has been

extensive research on the SG9R vaccine internationally, this is not the case for the local OBPft vaccine.

The main objective of this study was to gauge the efficacy, duration of immunity and safety of this vaccine when administered via the oral route using a reproducible experimental challenge model.

1.1.1 Objectives

The following specific objectives were therefore pursued:

- To develop an effective and reproducible challenge model for fowl typhoid.
- Test the safety of OBPft vaccine via injected and oral applications.
- Test the shedding of the vaccine strain in the safety trial and the challenge strain in the challenge trial.
- Test whether the OBPft vaccine would prevent fowl typhoid in commercial layer hens vaccinated via oral administration of 1ml of vaccine (1x 10^5 cfu) at 10 and 14 weeks of age.
- Test the duration of protection against fowl typhoid offered to commercial layer hens, by the OBPft vaccine using both the injected and oral routes of vaccination.

1.1.2 Hypothesis

H_1 – The disease model will cause 100 % mortality in susceptible chickens.

H_2 – The vaccine will cause no mortalities in immunized hens, irrespective of the route of administration.

H_3 –There will be no shedding of the SG vaccine strain through the eggs or faeces in the vaccinated groups, irrespective of the route of administration.

H_4 – The OBP vaccine will prevent fowl typhoid in chickens vaccinated via oral administration of 1ml of vaccine ($1x10^5$ cfu) at 10 & 14 weeks of age.

H_5 – The OBP vaccine will protect layers against fowl typhoid for at least 6 months post-vaccination.

1.2 LITERATURE REVIEW

1.2.1 Introduction

A serious septicaemic disease of primarily adult chickens and turkeys was recognised by Klein, in 1889 and the aetiological agent was called *Bacillus gallinarum.* The name fowl typhoid was applied in 1902 (Shivaprashad, 1997). Rettger first described the aetiological agent of pullorum disease in 1899, which was then called either bacillary white diarrhoea or fatal septicaemia of young chicks dependent on the clinical signs. In 1909 the organism was named *Bacterium pullorum* (referred to by Shivaprashad, 1997). The genus name for both bacteria was later amended to *Salmonella enterica* serovar Gallinarum in the 1984 edition of Bergey's Manual of Systemic Bacteriology. Serovar Gallinarum has been divided into two biovars: *S.e. gallinarum* and *S.e. pullorum*, causing fowl typhoid and pullorum disease, respectively (Le Minor, 1984).

1.2.2 Aetiology

Under the family Enterobactericae, the genus *Salmonella* is a facultative intracellular pathogen causing localised or systemic infections, as well as a chronic asymptomatic carrier state (Shivaprashad, 1997).

Salmonella enterica serovar Gallinarum, belongs to the serogroup D_1 (Shivaprashad, 1997; Le Minor, 1984). In addition to *S.* Gallinarum-pullorum, other important salmonellae serotypes such as *S.* Enteritidis*, S.* Panama and *S.* Dublin also belong to this serogroup (Le Minor, 1984). *Salmonella gallinarum* (SG) and *S. pullorum* (SP) cannot be distinguished serologically as they do not contain flagella antigens (H-antigens), but are divided into the two distinct biovars by biochemical tests (Christensen, Olsen, Hansen and Bisgaard, 1992).

They are Gram-negative, rod shaped, non-sporogenic, non-motile and highly host adapted pathogenic avian bacteria (Kwon, Park, Yoo, Park, Park and Kim, 2000). The biochemical reactions useful in differentiating SG and SP are shown in Table 1 below.

Table 1: Biochemical reactions to differentiate SG and SP

Reactant	*S. gallinarum*	*S. pullorum*
Dextrose	Fermented without gas	Fermented with gas
Mannitol	Fermented without gas	Fermented with gas
Maltose	Fermented without gas	Usually not fermented
Dulcitol	Fermented without gas	Not fermented
Ornithine decarboxylase	Not fermented	Fermented

A polymerase chain reaction followed by electrophoresis has also been used to identify isolates of SG and SP (Oliveira, Santos, Schuch, Silva, Salle and Canal, 2002; Oliveira, Rodenbusch, Cé, Rocha and Canal, 2003; Tayfun and Caner, 2001; Way, Josephson, Pillai, Abbaszadegan, Gerba and Pepper, 1993).

1.2.3 Sensitivity to chemical and physical agents

SG may survive for several years in a favourable environment, but is sensitive to heat, chemicals and adverse environmental factors. If heated to 60˚C it is killed within 10 minutes. In direct exposure to sunlight SG can last only a few minutes. SG is extremely susceptible to chemicals, and will die within a minute in 2 % formalin (Saif, 2003). SG has been found to retain viability for up to 43 days when subjected to daily freezing and thawing and has survived in liver specimens for more than 148 days at -20˚C even after been

thawed twice. SG can survive in faeces in a poultry house for more than 10 days. (Williams, Snoeyenbos and Pomeroy, 1984).

1.2.4 Distribution

Fowl typhoid has been largely eradicated from North America, Western Europe and Australia but in parts of South America, Asia and Africa the disease is still responsible for significant losses (Audisio and Terzolo, 2002; Barrow, Berchieri and al-Haddad, 1992; Pomeroy, and Nagaraja, 1991; Shivaprashad, 1997). In South Africa, there have been a number of outbreaks of the disease in layer flocks, particularly over the last two years. Currently the disease seems to be spreading rapidly between layer flocks (Bisschop, 2004). Fowl typhoid has also increased dramatically in parts of Latin America (Silva *et al*., 1981a). In areas where this disease is endemic, it has become one of the most important diseases of chickens (Silva *et al*., 1981a; Bouzoubaa, Nagaraja, Newman and Pomeroy, 1987), and occurs in both backyard chickens and in commercial poultry (Harbourne, 1957; Saif, 2003).

1.2.5 Host affected

Salmonella enterica is a facultative intracellular pathogen that is capable of causing disease in a wide range of hosts (Gast, 1997). Although more than 2 300 serotypes of *Salmonella* have been identified, only about 10% of these have been isolated from poultry (Gast, 1997). Chickens are the natural hosts for the highly host adapted biovars SG and SP, but natural outbreaks have been reported in turkeys, Guinea fowl, quail and pheasants (Pomeroy and Nagaraja, 1991; Saif, 2003). The lighter breeds, particularly Leghorns, are more resistant to infections than the darker breeds, for example Rhode Island Reds (Silva *et al*., 1981a). A major problem in experimental trials has been that Leghorns have been used. Mortalities in

these birds are low, which makes results difficult to interpret. (Silva *et al*., 1981a; Bumstead and Barrow, 1993; Gast and Beard, 1990; Smith and Tucker, 1980).

Silva, Snoeyenbos, Weinack and Smyser, (1981b) concluded from extensive studies on the use of the SG9R vaccine strains in white leghorns, meat type and brown layer hens that protection against mortality in highly susceptible stock exposed to virulent strains may be severely limited.

1.2.6 Age

Fowl typhoid affects mostly adult chickens (Shivaprashad, 1997), although it can occasionally result in high morbidity and mortality in young chicks from a few days old (Hall, Legenhausen and Macdonald, 1949; Saif, 2003). Disease and lesions in chicks are indistinguishable from those associated with pullorum disease (Shivaprashad, 1997). A certain percentage of chicks that survive the initial infection are said to become carriers in their feather dust, faeces, mucosal secretions and may have misshapen discoloured cystic ova, with or without the presence of clinical signs and pathological lesions (Pomeroy and Nagaraja, 1991; Shivaprashad, 1997).

1.2.7 Transmission

Fowl typhoid is transmitted by either horizontal or vertical transmission (Berchieri, Murphy, Marston and Barrow, 2001). The primary role of infected hatching eggs in the transmission of these two diseases was recognised in the early course of investigations. The chickens may be exposed to fowl typhoid at a very young age without showing any clinical signs, but they can harbour infection until they come into lay, then they can produce infected eggs and infected progeny (Wigley, Hulme, Bumstead and Barrow, 2002; McIlroy, McCracken, Neill and O'Brien, 1989). SG may also enter cracked eggs at any stage before hatch (McIlroy *et*

al., 1989). The most common route of transmission is by the ingestion of contaminated feed and water and faeces. Transmission may also occur in a flock as a result of cannibalism of infected birds, infected egg eating, via infected skin wounds or via mucosal discharges (Shivaprashad, 1997). Under favourable conditions SG can persist in the faeces for at least one month and in infected carcasses for up to two weeks (Wray, Davies and Corkish, 1996).

Attendants, feed dealers, chicken buyers and visitors who move from house to house or farm to farm may carry infections unless precautions are taken to disinfect footwear, hands and clothing (Snoeyenbos, 1991). Contaminated crates and trucks may be involved in the transmission of this disease, although not as much as for SE (Christensen, Skov, Hinz and Bisgaard, 1994). Wild birds, rodents and flies may be important mechanical spreaders of the organisms (Shivaprashad, 1997).

1.2.8 Pathogenicity and immunity

The pathogenicity of *Salmonella* depends on the invasive properties and the ability of the bacteria to survive and multiply within the cells, particularly macrophages. The principle site of multiplication of these bacteria is the digestive tract, which may result in widespread contamination of the environment due to bacterial excretion through faeces. The bacteria invade through the intestinal mucosa, caecal tonsils and Peyer's patches. They are within a local immune compartment known as the mucosal immune system and more specifically the gut-associated lymphoid tissue (GALT).

There are two key features that define this compartment. Firstly, the immune response induced within the compartment is largely confined in expression to that compartment.

Secondly, lymphocytes are restricted to particular compartments by expression of homing receptors bound by ligands and they are specifically expressed within the tissues of the compartment. The mucosal surfaces are particularly vulnerable to infection. The Peyer's patches have specialised epithelial cells with microfolds on their luminal surface, and are known as microfold cells (M cells) (Janeway, Travers, Walport and Shlomchik, 2001). These cells take up foreign material, including bacteria from the intestinal lumen by endocytosis or phagocytosis and transport them to underlying antigen-presenting cells. These cells in turn migrate to the local lymphoid tissue and present the foreign material to the lymphocytes. Depending on the pathogenicity of the organism and the strength of the host adaptive immune response, infections that breach the intestinal mucosa may be cleared with minor tissue damage, cause a local inflammatory response or spread further. Salmonellae that are able to survive in these cells and are transported via the lymphatic system to organs rich in reticulo-endothelial tissues, such as the liver and spleen (Janeway *et al*., 2001). These two organs are the main sights of multiplication of salmonellae (Barrow, Huggins and Lovell, 1994).

When inadequate body defence mechanisms occur, secondary invasion of the bloodstream occurs and the bacteria localize in other organs, particularly the ovary, oviduct, myocardium, pericardium, gizzard or lungs (Bumstead, and Barrow, 1993; Barrow, 1993). The primary virulence factor of SG is bacterial lipopolysaccharide (LPS) which is involved in the development of anaemia, hypersensitivity reactions and ultimately death.

Anaemia may be observed in both acute and sub-acute forms of fowl typhoid infections and the severity of the haematological changes appear to correlate with the number of bacteria involved (Assoku and Penhale, 1974). Haemolytic anaemia is induced by LPS and LPS-

antibody complex. *Salmonella pullorum* does not cause anaemia (Assoku and Penhale, 1974).

Buxton and Davies (1963) used bacterial agglutination, and antiglobulin HA tests to detect antibody production during the development of SG infection in chickens. The antiglobulin HA test detected serum antibodies as early as 1 day after oral infection, and antibodies were detected in all birds at the time of death. An accumulation of bacterial polysaccharide in tissues of infected birds was detected by an HA test. Of the chickens that died of the disease, different organs had variable but high concentrations of this bacterial polysaccharide. In birds that recovered from an acute infection, the concentrations were low or undetectable. Buxton and Davies (1963), postulated that an antigen-antibody reaction that developed as an anaphylactic type of hypersensitivity may in fact be closely related to the production of clinical signs and death of chickens infected with SG.

The dominant antibody isotype of the mucosal immune system is IgA in the dimer form. Polymeric immunoglobulin A (IgA) and IgM are synthesised throughout the intestine and are transported to the luminal surface by transcytosis, from here the IgA and IgM antibodies are secreted and bind to the mucous layer overlying the intestinal epithelium where they can bind to and neutralise intestinal pathogens. The IgM molecule is the first to be produced when a foreign organism is recognised and has a better ability to agglutinate antigen than IgG, as it has 10 binding sites. Salmonellae cause a brisk local and systemic inflammatory response associated with the T helper cell and antibody responses of the IgG and IgA classes (Collins, 1974; Janeway *et al*., 2001). Allan, Duffus and Higgins, (1968) found following an oral live SG infection, that serum collected on the 9th day post challenge and tested for the presence of haemagglutinating antibody, showed a marked increase in

IgG. The immune response associated with chickens developing acute and subacute clinical signs, showed that an unusually high concentration of incomplete IgM antibodies were found in acute infection. In subacute infection, however, neither complete nor incomplete IgM specific antibodies were found to correlate with clinical signs. Using a bactericidal assay for the presence of antibody, Horsfall, Rowley and Jenkin, (1970) found that 1-day-old chicks had little natural antibody against SG, even though adults possessed relatively higher levels.

1.2.9 Clinical signs

Mortality in adults may vary from 10 – 90 % (Williams, *et al*., 1984; Hall *et al*., 1949), whereas in chicks it can be up to 68% (Lowry, Tellez, Nisbet, Garcia, Urquiza, Stanker and Kogut, 1999).

Fowl typhoid can appear in peracute, acute or chronic forms. In the peracute form, the birds may die without showing any noticeable clinical signs (Williams, *et al*., 1984). In growers and adults, a watery to mucoid yellowish diarrhoea is the most characteristic clinical sign in the acute phase of the disease (Gast, 1997). In the chronic form of the disease, severe anaemia is the predominant sign (Assoku and Penhale, 1974). In addition to the anaemia; progressive loss of body weight, reduced feed consumption and egg production, ruffled feathers, shrunken pale combs and wattles are characteristic signs (Wray *et al*., 1996). The chicks exhibit lassitude, huddle together, have droopy wings, pasted vent, laboured breathing and a distorted body appearance (Williams *et al*., 1984; Gast, 1997). SG reduces the laying capacity by up to 50 % and hatchability by as much as 15% (Shivaprashad, 1997).

1.2.10 Pathological lesions

SG can produce lesions in chicks which are indistinguishable from those associated with pullorum disease, namely; enlarged and congested liver, spleen and kidneys (Pomeroy *et al*., 1991). In peracute cases, the organs are severely congested without major pathological changes, due to mortality occurring so soon after infection. In acute cases, the most common changes are septicaemia, enlarged liver and spleen with necrotic foci. The liver usually turns a shiny bronze colour (Pomeroy *et al*., 1991). Gross lesions due to fowl typhoid in chicks and poults include hepatitis, splenitis, typhlitis, omphalitis and peritonitis (Saif, 2003). In mature fowl, lesions include oöphoritis, salpingitis, orchitis, peritonitis and perihepatitis (Shivaprashad, 1997). Misshapen, discoloured, pedunculated, cystic ova with various forms of pericarditis and peritonitis, swelling of the hock joints and nodular heart muscles resembling the neoplastic disease, are frequently evident in chronic cases (Wray *et al*., 1996).

1.2.11 Histopathology

In peracute cases of fowl typhoid only severe vascular congestion in various organs, especially liver, spleen and kidney can be identified. In acute to subacute cases, multifocal areas of necrosis of the hepatocytes with the accumulation of fibrin and the infiltration of heterophils in the hepatic parenchyma can be observed. Periportal infiltration of heterophils mixed with a few lymphocytes and plasma cells can also be seen in the liver. Chronic cases, especially in cases in which there are large nodules in the heart, the liver will have chronic passive congestion with interstitial fibrosis. The spleen may have severe congestion or fibrin exudation of vascular sinuses in acute stages and severe hyperplasia of the mononuclear phagocytic system cells in later stages. The caeca in young chicks may have extensive necrosis of the mucosa and sub- mucosa, with an accumulation of necrotic debris

mixed with fibrin and heterophils in the lumen. The most characteristic microscopic lesions are in the heart and ventriculus. In the heart they begin as necrosis of myofibers with infiltration of heterophils mixed with lymphocytes and plasma cells. In the later stages, these cells are replaced by massive numbers of uniform histiocytes. These cells are fairly large, with irregular vesicular nuclei and faintly staining, foamy eosinophilic cytoplasm. They may be arranged in solid sheets, forming nodules that often protrude from the epicardial surface. These nodules, both grossly and histologically, can be confused with lymphoid tumours caused by Marek's disease and possibly retroviruses. A similar process can be seen in the ventriculus and pancreas. The lesions in the pancreas can be so severe that the normal structure is destroyed (Saif, 2003; Shivaprashad, 1997).

Other changes, such as serositis of various organs including the pericardium, pleuroperitonium, synovium and serosa of the intestinal tract and mesentery can be seen in a high percentage of cases. In acute stages, these lesions can be associated with heterophils and fibrin, but in the later stages, only lymphocytes, plasma cells and histiocytes are visible. Microscopic lesions in the ovary range from acute fibrinosuppurative inflammation to severe pyogranulomatous inflammation of the ovules. The pyogranulomatous inflammation is characterised by infiltration of heterophils mixed with fibrin and bacterial colonies in the coagulated yolk material. In turn, the core is surrounded by successive layers of multinucleated giant cells and a mixed population of inflammatory cells that can include macrophages, plasma cells, heterophils and lymphocytes. In males, degeneration, necrosis and inflammation of the epithelial cells lining the seminiferous tubules can be seen. Other less common changes are; catarrhal bronchitis, catarrhal enteritis and interstitial inflammation of the lung and kidneys (Saif, 2003; Shivaprashad, 1997).

1.2.12 Diagnosis

Fowl typhoid cannot be differentiated clinically or pathologically from other systemic diseases. Serology has only been found to be useful when monitoring for the disease in unvaccinated animals and to provide a rapid presumptive diagnosis (Snoeyenbos, 1987; Wray *et al*., 1996). Antibodies are not elevated in peracute disease and some infected birds do not mount a serological response. The tests are also not specific enough, and the different technicians can use different interpretation of agglutination in the plate tests. A definitive diagnosis depends on the isolation and biotyping of SG. The specimens of choice are liver, spleen, caecal tonsils and GALT. The Polymerase Chain Reaction (PCR) can be used to identify salmonellae that commonly infect chickens both in specimens as well as from cultures. In a trial done by Tayfun, and Caner, (2001) 38 strains of salmonellae were tested both by PCR and culture. In that trial the PCR successfully identified all the salmonellae samples.

1.2.13 Treatment

Reasonably effective prophylactic and therapeutic drugs have been developed to help treat SG and SP. Various sulphonamides, nitrofurans, amphenicols, tetracyclines and aminoglycosides have been effective in reducing mortality from fowl typhoid and pullorum disease (Saif, 2003). No single antibiotic or antibiotic combination has, however, been able to eliminate infection from a treated flock (Williams *et al*., 1984). Some SG strains have proven to be resistant to furazolidone (Stuart, Keenum and Bruins, 1963; Stuart, Keenum and Bruins, 1967).

1.2.14 Prevention and control

Successful eradication or disease-free programmes can be achieved by developing good hygiene and management together with routine serological tests and slaughter policy (Barrow, 1993). Freedom from SG entails acquiring chicks free from infection, and the chicks should be placed in a cleaned, sanitised and SG free environment with strict biosecurity measures (Pomeroy *et al*., 1991). The feed and water should be free of salmonellae species contamination. Dead birds need to be properly and promptly disposed of. Adequate precautions are needed to prevent infections from mechanical carriers like footwear, human clothing, hatchery disciplines, equipment, litter, crates, trucks and processing plants (Christensen *et al*., 1994). Wray *et al.* in 1996 described that the birds need to be tested at the age of 16 weeks due to immunologic maturity, at the point of lay due to stress, this should be done twice, one month apart to provide the acceptable evidence that the flock is free from fowl typhoid (Wray *et al*., 1996; Barrow, 1993). Where the above cannot be fully implemented, vaccination and treatment of disease by the use of antibiotics can be done.

Killed vaccines are of little value in SG control, as they fail to control intestinal colonization (Silva *et al*., 1981b) and provide an adequate cellular immunity. Nobilis SG9R vaccine has been used subcutaneously either from broth culture, (Gordon, Garside and Tucker, 1959a; Gordon, and Luke, 1959b; Harbourne, 1957) or from a reconstituted freeze-dried state (Harbourne, 1963). Oral vaccination of Nobilis SG9R only gave 60 % protection and produced more hepatic and splenic lesions than the subcutaneously injected group, which had 80 % protection (Bouzoubaa, Nagaraja, Kabbaj, Newman and Pomeroy, 1989; Silva *et al*., 1981a). The SG9R vaccine does not limit egg transmission of SG, as the challenge strain was isolated from eggs of birds vaccinated subcutaneously and later challenged.

Vaccinated birds appear to be capable of transmitting the SG9R vaccine and pathogenic strains through the egg. Although the vaccine strain is rough, some birds develop antibodies that produce reactions in serological tests for SG (Silva *et al*., 1981a; Silva *et al*., 1981b).

Vaccination of adult birds with a rough strain of SG may induce pathological changes in the ovary of some birds (Gordon, *et al*., 1959b). Potential egg transmission of the SG9R strain following vaccination and of a pathogenic strain following challenge of vaccinated birds was indicated by ovarian infection with each strain and by isolation of the pathogenic strain from eggs (Silva *et al*., 1981a). Distorted ovules or diseased ovaries were found in 6/25 birds vaccinated with the SG9R strain (Harbourne, 1957). Harbourne (1963) also recovered the vaccine strain from two unvaccinated sentinel birds in a flock of SG9R vaccinated birds. This confirms the Gordon, *et al*. findings in 1959 that under certain conditions there may be a transfer of the 9R vaccine strain from vaccinated to in-contact birds. There have been conflicting results on the potential spread of the SG9R strain from vaccinated to unvaccinated birds, and lesions found in the liver and spleen of birds vaccinated by the SG9R vaccine (Bouzoubaa *et al*., 1989; Feberwee, Hartman, de Wit and de Vries, 2001), shows that this vaccine has room for improvement.

CHAPTER 2

ESTABLISHING AN EFFECTIVE SG CHALLENGE MODEL

Introduction

In order to test the level of protection and duration of immunity of the OBPft vaccine by the oral route, it was essential that a challenge model resulting in 70 – 100 % disease in susceptible birds be developed (Silva *et al*., 1981a). Although there are published challenge models for salmonellosis in chickens, the outcome of infection is dependent on the route of infection, the virulence of the strain used, the infectious dose and host susceptibility (Silva *et al*., 1981a; Shivraprashad, 1997). The first pilot trial was done to select the most virulent strain of SG from a few smooth isolates obtained from the Poultry Reference Centre and the second one was done to find the optimum challenge dose of the most virulent bacterial strain.

2.1 SELECTION OF A HIGHLY VIRULENT SG STRAIN

In this pilot study commercial layer hens were challenged with wild-type SG strains 318/03 and 1737/03 isolated by the Poultry Reference Centre at Onderstepoort from past SG outbreaks in South Africa, as well as an American type culture collection SG strain (ATCC 9148). The susceptibility of the brown commercial layer and white leghorns to SG was also compared. This was to test the hypothesis that the latter were more resistant to infection and are thus not suitable for use as challenge birds.

2.1.1 Materials and methods

Preparation of the challenge strains

The 3 challenge strains that had been stored in brain heart infusion broth (BHI) at –86 °C were rapidly defrosted and plated onto 7 % Blood Columbia (BCA) and MacConkey agars (MAC) (Oxoid Pty Ltd, Basingstoke, England) and incubated overnight, in air, at 37 °C. The cultures were then checked for purity and the isolates re-identified using a commercial biochemical test profile, API 10S (BioMerieux, France) (Proux, Humbert, Jouy, Houdayer, Lalande, Oger and Salvat, 2002).

A colony of each of the challenge bacteria was added to separate test tubes containing 5 ml of BHI and incubated at 37˚C for 24 hours. The optical density index (ODI) of the cultures were read in a spectrophotometer (Ultrospec II, LKB Biochrome) at a wavelength of 540nm and adjusted to as close to 0.3 as possible using dilutions of BHI as indicated in Table 2. Viable bacterial plate counts were done on the selected dilutions of each bacterium (O.I.E., 1992). A 1 in 10 dilution was made using 1.5 ml of the bacterial suspension and diluting it in 13.5 ml of BHI. Further 1 in 10 dilutions were made by adding 0.1 ml of the bacterial suspension to 0.9 ml of Normal saline and vortexing the sample. This procedure was repeated a further eight times. The entire procedure was repeated to minimize dilution errors. 0.1 Millilitres of each dilution were then spread-plated onto BTA and MAC and incubated overnight at 37 °C. Colony counts were done on those dilutions where there were between 10 and 50 bacterial colonies. The results of each count was multiplied by the dilution factor, with the counts of each isolate being added together and then divided by the number of counts done per isolate. The average bacterial count per isolate was expressed in colony forming units (cfu). The results are listed in the last column of Table 2.

Table 2: Culture mixtures and spectrophotometer readings

Strain	BHI (ml's added)	Bacterial suspension (ml's added)	ODI readings @ 540nm	Dose (cfu)
318/03	2.5	0.5	0.246	
318/03	2.5	0.6	0.285	
318/03	2.5	0.65	*0.302	$1x10^7$
1737/03	2.5	0.5	0.214	
1737/03	2.5	0.6	0.252	
1737/03	2.5	0.7	*0.279	$3.5x10^7$
ATCC 9184	2.5	0.5	0.174	
ATCC 9184	2.5	0.6	0.197	
ATCC 9184	2.5	0.9	*0.277	$0.5x10^7$
Blank	1	0	0	

* Chosen bacterial suspension from each strain.

Challenge

Six vaccinated (intramuscularly) and six non-vaccinated hens (3 commercial brown hens and 3 leghorns) were placed in each group and were challenged with the different strains as indicated in Table 3. Seven unvaccinated Leghorns were used as unchallenged negative controls. All the birds in this pilot trial were around 30 weeks old and were in lay.

The hens were maintained in a fitted out layer facility with three birds per cage and fed layer ration *ad libitum*. Water supply was also made available *ad libitum.* The same challenge groups were kept together for the duration of the trial. The trial duration was 14 days. The agglutination test for SG (BWD rapid serum plate agglutination test, Intervet), as well as pooled faeces, cloacal swabs and egg culturing was done prior to challenge and just before euthanasia on day 14.

One millilitre of each bacterial suspension was administered *per os* at the beginning of the trial to the birds as indicated in Table 3. Thereafter the birds were observed twice daily for any clinical signs of salmonellosis and those birds that died were removed and necropsies

were performed. The trial was terminated 14 days post-challenge, the surviving birds were euthanased and full necropsies were performed on all the birds to test severity of the challenge. All the birds in this trial were euthanased by lethal injection of 0.1 ml sodium pentobarbitone, (Eutha-nase, Centaur Bayer) administered intracardiacally. All carcasses were then securely bagged and incinerated at the Onderstepoort Veterinary Institute incinerators.

Culturing of specimens

a) Pre enrichment (day 1):

Faecal specimens were mixed well, and 25 g was packed in Whirl Pak bags and 250 ml of buffered peptone water (BPW) was added and mixed. Cloacal swabs were placed in tubes containing 10 ml BPW and incubated at 37 °C for 24 hours. Three eggs were placed in one Whirl-Pak bag (Guth. NDE SA), the shells were crushed carefully to release the content and 100 ml BPW was added. Approximately 10 g of organs was collected and placed in a labelled Whirl-Pak bag. The bag with organs was then placed in a stomacher (Lab blender 400) and homogenised for 1 minute and 100 ml of BPW was added. After having been mixed well, the bags were placed in a wire basket and incubated at 37 °C for 24 hours.

b) Selective enrichment (day 2):

One millilitre of pre-incubated BPW was transferred to 9 ml Rappaport broth using a micropipette, and incubated at 41.5 °C for 24 and 48 hours.

c) Selective isolation (day 3 and 4):

A xylose lysine deoxycholate medium (XLD) and MAC agars was streaked with 0.1 ml of pre-incubated Rappaport broth and incubated at 37 °C for 24 hours.

d) Examination of plates (day 4 and 5):

Plates were examined after 24 and 48 hours of incubation. MacConkey plates were then examined for any pale non-lactose fermenting colonies and suspected colonies and XLD for clear black colonies. The selected colonies were subcultured onto MacConkey plates and incubated at 37 °C for 24 hours.

e) Identification (day 5 and 6):

The purified cultures, if non-lactose fermenters, were identified with the API 10S system (API 10S, BioMerieux, France) (Proux *et al*., 2002; Saif, 2003). A malonate fermentation test was done to exclude salmonellae Group II (Proux *et al*., 2002). They were incubated at 37 °C for 24 hours. Subcultures were made of the pure cultures onto BTA and swarming agars and incubated at 37 °C for 24 hours.

Necropsies

Full necropsies were done on those that died and on the rest of the birds after euthanasia at the end of the trial (Saif, 2003). Specimens were taken from the liver, spleen, lung and kidney for bacteriological culture. Any pathological changes were noted and compared with the pathological changes expected from birds infected with SG.

Serum was collected from all birds, before challenge and pre-euthanasia, if the birds had died they were bled from the heart where possible.

2.1.2 Results, Discussion and Conclusion

Table 3: The 1st pilot study trial plan and mortality rate.

SG Strain	Mortality Rates (dead/total in group)			Challenged		Challenge Dose
	Vaccinated Commercial Hens	Unvaccinated Commercial Hens	SPF Leghorns	Yes	No	X 10^7 cfu.
ATCC 9148	0/6	0/3	0/3	12	-	0,5
318/03	3/6	1/3	0/3	12	-	1
1737/03	0/6	0/3	0/3	12	-	3.5
Neg. control	None	None	0/7	-	7	-

The mortality rates are shown in Table 3. The 318/03 strain was the only strain to kill both vaccinated and unvaccinated birds. The unvaccinated birds died 5 – 7 days post-challenge and the vaccinated birds died 7 – 10 days post-challenge.

Prior to challenge no SG could be isolated from any of the specimens taken from the birds and neither could any antibodies be detected. SG was not cultured from any of the egg contents nor from cloacal swabs of birds taken just prior to euthanasia. The culturing and serology results are shown in Table 4.

Table 4: SG challenge test results

SG Strain	Pooled faecal swabs	Organ swabs	Serology	
			Pre-Challenge	Pre-euthanasia
	Pos.	**Pos.**	**Pos.**	**Pos.**
ATCC 9148	1/4	0/12	0/12	11/12
318/03	0/4	4/12	0/12	12/12
1737/03	3/4	0/12	0/12	12/12
Neg. control	0/3	0/7	0/7	6/7

Antibodies were detected in most of the birds just prior to euthanasia. Some of the birds challenged with the ATCC and 1737/03 strains had positive faecal cultures. The only positive organ swabs were found in the birds that had died after challenge with the 318/03 strain, in each case the liver, spleen and lung swabs were positive for SG colonisation. Pre-euthanasia all, except two birds, were serologically positive for SG. Six out of seven negative controls sero-converted during this trial.

The results indicate that 318/03 was the most virulent and invasive strain as it was the only one that resulted in mortalities and invaded the organs. Vaccination in this case seemed to have no beneficial effect on survival rates. The vaccinated birds were 30 week-old birds that had been vaccinated at 8 and 12 weeks-old with the Intervet SG9R vaccine. The birds were thus challenged 18 weeks after vaccination which is 6 weeks longer than the estimated duration of protection of the Intervet SG9R vaccine. Although all the white Leghorns sero-converted none died, nor could SG be cultured from them. Thus they seem to be more resistant to disease.

Pooled faecal samples had a higher sensitivity than cloacal swabs. This could be due to the small specimen size, where mucus rather than faeces was trapped on the cloacal swabs. The challenge dose was a little lower than recommended in previous literature; we aimed for 1 x 10^8 cfu per dose which is the dose used by Silva *et al* (1981a). Here a dose of $1x10^7$ cfu's was used, this dose was 10 fold weaker than that used by Silva *et al* (1981a), and we had a lower killing rate than that achieved by them, but that was expected as some of our birds were leghorns and some were vaccinated. Liver and spleen swabs were good indicators of SG infection as all the liver and spleen swabs taken from the birds dying due to challenge were positive for SG colonisation. Contrary to expectation, SG was not

cultured from the eggs. However, eggs were only harvested for culture 14 days post-challenge, it is possible that they were no longer bacteraemic, therefore no shedding would occur. Most birds in this trial sero-converted, even the negative controls that were neither vaccinated nor challenged. This shows that SG spreads horizontally, as expected (Gast, and Beard, 1997; Feberwee *et al*., 2001).

2.2 DETERMINATION OF CHALLENGE DOSE AND PATHOLOGICAL EFFECT OF VACCINATION ON RECENTLY INFECTED BIRDS

2.2.1 Introduction

Three challenge groups of 6-week-old commercial brown layer hens were challenged with either of the following; $1x10^8$, $1x10^9$ or $1x10^{10}$ cfu's per ml of SG strain 318/03. Some birds were vaccinated 3 days post-challenge to test the effect of the vaccine on already infected birds. This was done to determine the effect of vaccination on infected birds. In this pilot trial 6-week-old birds were used as no unvaccinated adult birds were available, and there was insufficient time allowed for the birds to age.

2.2.2 Materials and methods

Culturing and calculation of dose

Buffered peptone broth was added to an overnight culture of SG isolate 318/03 to obtain the spectrophotometer ODI readings at a wavelength of 540 nm. This is shown in Table 5. Bacterial counts were done in duplicate using the method described in section 2.1.1. A ten-fold dilution was made of the bacterial suspensions 1 and 2 and a 5-fold dilution of bacterial suspension 3 was made to obtain the final required inoculums as shown in Table 5.

Table 5: Culture mixtures and ODI readings for challenge dose calculation

Challenge inoculum number	BHI media (ml)	Bacterial suspension (ml)	ODI	Dilution	Dose (cfu)
1	2	1	0.412	10x	$3x10^8$
2	1.7	1.3	0.514	10x	$1.5x10^9$
3	1.5	1.5	0.567	*5x	$1x10^{10}$

* Changed dilution factor to achieve goal dose

Housing

Birds were housed in standard layer cages with 5 birds per cage and were fed layer ration *ad libitum*, water was also supplied *ad libitum*.

Inoculation of birds

Six-week-old commercial brown layer hens were placed in two groups. Each group had three sub-groups each consisting of 5 birds. The group 1 birds were challenged with 1 ml of 318/03 SG challenge strain *per os* at 6-weeks of age, and vaccinated with the SG9R vaccine three days later. The different sub-groups of group 1 were challenged with different doses of the same challenge strain (Table 6). Group 2 birds were challenged in the same way as Group 1; however they were not vaccinated at all. All survivors were euthanased two weeks after challenge.

Table 6: 2nd pilot challenge trial plan

Group	No.	Challenge Dose (cfu)	Challenge at 6 weeks of age	Vaccinated 3 days later	*Euthanased at 8 weeks of age
1A	5	10^8	5	5	Survivors
1B	5	10^9	5	5	Survivors
1C	5	10^{10}	5	5	Survivors
2A	5	10^8	5	0	Survivors
2B	5	10^9	5	0	Survivors
2C	5	10^{10}	5	0	Survivors

*If not euthanased prior, due to signs of illness.

Other procedures

Pooled faecal specimens were taken from the cages of each group 48 hours post-challenge and 24 hours after Group 1 was vaccinated, the samples were then cultured for SG using the enrichment method previously described. Necropsies and organ cultures for SG were

done as previously described. In addition body cavity swabs were also taken for bacterial culture. Haematocrits were determined, pre-challenge, 3, 5 and 7 days post challenge.

2.2.3 Results, Discussion and Conclusion

Cultures

The 1C, 2B and 2C groups were positive for SG at the 48-hour post challenge sampling and 1B, 1C, 2B and 2C groups were positive for SG 24 hours after group 1 was vaccinated (Table 6). SG was isolated from the pooled faeces of groups 1C, 2B and 2C, 48 hours post-challenge and groups 1B, 1C, 2B and 2C after a further 48 hours. It was also isolated from all the sampled organs of all the birds and two of the body cavity swabs of birds in Group 1B. This indicated that the shedding of bacteria in faeces may be associated with the infectious dose as SG was not cultured from the faeces in groups 1A and 2A. The haematocrit values were constant over all the test times, no anaemia was observed. The last test was done seven days post challenge, this was also the day that produced the most mortalities. Anaemia is described as part of the chronic form of the disease, and considering that the mortalities expected and observed are more acute this test was no longer pursued.

Mortality rates

Table 7: 2nd pilot challenge, mortality rates

Groups	Mortalities days post challenge							% mortality
	5	6	7	8	9	10	11	
1A			1		1			2/5 = 40%
1B	1		2	1	1			5/5 = 100%
1C			1	1	1			3/5 = 60%
2A			1	1				2/5 = 40%
2B		1	1		1			3/5 = 60%
2C		1	1	1			1	4/5 = 80%

It was difficult to assess the effect of vaccination due to the small number of birds in each group and the fact that SG could be isolated from all the birds. It was expected that the mortality rate would increase in those birds vaccinated post challenge, as a live vaccine was used thus adding to the challenge dose already given. However this only occurred in the B groups where there was a 40 % mortality increase in the vaccinated group, when compared to the unvaccinated group (Table 7). The opposite occurred in the C groups where there was a 20 % increase in mortality in the unvaccinated group; this could be due to the high number of bacteria given in the challenge, thus vaccination did not increase the count much. The numbers used in this pilot study are small so the percentages are probably exaggerated.

Groups with the same challenge dose were combined in order to get percentage mortality per challenge dose; this would help decide which dose would be best suited for the project. From groups 1A and 2A the birds were challenged with $1x10^8$ cfu's and all together only 40 % of the birds died. From groups 1B and 2B the birds were challenged with $1x10^9$ cfu, in this group all together 80 % of the birds died. From groups 1C and 2C the birds were challenged with $1x10^{10}$ cfu all together 70 % died (Table 7).

Due to the higher mortalities caused by SG infection found in Groups 1B and 2B, compared with the other groups, it was decided that $1x10^9$ was the best challenge dose to use.

CHAPTER 3

MATERIALS AND METHODS

Broad trial design

Forty-three commercial brown layers were used for the safety trial. The birds were split randomly into groups. There were 10 birds per group, except for the negative control that had only 3 birds (Table 8). The groups differed by method and number of vaccinations.

Ninety commercial brown layers were used for the duration of protection trial. Birds were divided randomly into 3 groups, a group vaccinated orally at 10 and 14 weeks of age, a group vaccinated via intra-muscular injection at 10 and 14 weeks of age and a control group, not vaccinated at all (Table 11). These groups were split further into groups of 10 that were challenged at different time intervals post-vaccination.

All the one hundred and thirty three day-old commercial type brown layers were placed together at day-old in isolated rearing cages. The birds were fed *ad libitum* throughout the trial. At 40 days of age the birds were randomly assigned to treatment groups. This was done by placing the birds in a large closed box and without looking removing birds one by one from the box. Then random numbers were used to decide which group each bird would be placed in.

The OBPft vaccine was used, at a dose of 1 x 10^5 cfu's per ml. The birds vaccinated by intra-muscular injection were injected with 1ml of vaccine into the breast muscle, alongside

the sternum using a 1ml insulin syringe with a 23-gauge needle. The birds vaccinated orally were given 1ml of vaccine directly into the mouth with a 1ml insulin syringe.

3.1 SAFETY TRIAL

In order to determine the safety of the OBPft vaccine, forty Hyline Brown hens (Hyline, South Africa) were vaccinated. The hens were randomly divided into four treatment groups as indicated in Table 8 below. Group 1 was vaccinated at 10 and 14 weeks of age by intramuscular injection. Group 2 was vaccinated at 10 and 14 weeks of age by oral gavage. Birds in groups 3 and 4 received a single vaccination at 14 weeks by intramuscular injection or oral gavage respectively.

Table 8: Treatment groups for OBPft safety trial

Group	Number of birds	10-week-old vac	14-week-old vac
1	10	Injected	Injected
2	10	Oral	Oral
3	10	-	Injected
4	10	-	Oral
Negative control	3	-	-

Two birds from each of the treatment groups were humanely euthanased and autopsied at two-day intervals starting two days after vaccination, as indicated in Table 9. The procedures were modified slightly from those of Silva *et al*., (1981a). The timing was changed and extended to give more information on the pathological effects of the vaccine on the birds.

Table 9: Euthanasia and testing schedule post vaccination for the safety trial

Days of euthanasia post-vaccination	2d	7d	14d	21d	28d
Groups	Number of birds				
1	2	2	2	2	2
2	2	2	2	2	2
3	2	2	2	2	2
4	2	2	2	2	2
Neg. control	0	1	1	1	0

In Table 10 there is a list of the tests performed. This list shows how many samples were taken at each time period post vaccination. These tests were done to determine which organs the OBPft vaccine strain colonises and how severely it affects the birds. The faecal swabs and intestinal scrapings were done to follow any shedding pattern that might be present.

The severity of the pathology was graded using a 1 to 3 scoring system. To ensure that there were no discrepancies in the assignment of the pathological grouping. All necropsies and thus assignment of pathology values was done by the same person throughout the entire project.

Key for organ pathology categorising used throughout the trial:

The organs tested (spleen, liver, lung and intestine).

1 = slight but visible changes in pathology from the norm

2 = easily notable changes in pathology from the norm

3 = gross lesions and/or vast changes in pathology

Table 10: Tests used to monitor safety trial

Tests	Pre vaccination	Post vaccination at 14 weeks-old				
		2d	7d	14d	21d	28d
Faeces Pooled	4	4	4	4	4	4
Serology	20	8	8	8	8	8
Liver swab	0*	8	8	8	8	8
Caecal tonsil scrape	0*	8	8	8	8	8
Mid-intestine mucosal scrape	0*	8	8	8	8	8
Spleen swab	0*	8	8	8	8	8

* - no samples taken.

3.2 DURATION OF PROTECTION TRIAL PROCEDURE

Sixty birds were vaccinated at 10 and 14 weeks of age using either the intramuscular or oral route. The different groups of birds were challenged orally with the 318/03 SG challenge strain at intervals of 8 weeks from the 14-week vaccination. The first group was challenged at 22 weeks old, the second at 30 weeks old and the third at 38 weeks old. There was also an unvaccinated control group (Table 11). The tests used in the duration of protection trial are shown in Table 12. These tests included faecal and egg samples to monitor the shedding of SG post-challenge. Eggs laid were also counted in each group on a daily basis to monitor egg production through the trial, and surviving birds were euthanased 2 weeks post-challenge. Necropsies and laboratory investigations were done on all birds, both those that died of disease and the euthanased birds, to detect SG pathology and the presence of SG in the organs. From this data it was possible to compare the duration of protection achieved by oral vaccination with that of intra-muscular injection.

Table 11: Challenge groups for duration of protection trial

Group	22 week-old challenge	30 week-old challenge	38 week-old challenge
Oral	10	10	10
Injected	10	10	10
Negative control	10	10	10

Table 12: Tests used in duration of protection trial per challenge

Tests performed	Pre challenge	Post challenge (days) 3	4	5	6	7	14
Faeces pooled	9	9	0	9	0	9	9
Serology	30	*	*	*	*	9*	Survivors
Eggs pooled	0	0	0	20	0	20	20
necropsy	0	#	#	#	#	#	Survivors

* Blood removed from heart of dead birds where possible, # Necropsies done on all deaths

3.2.1 Laboratory Procedures

Challenge sample

The challenge sample (318/03 Fowl Typhoid strain collected by the PRC at Onderstepoort) was added to 10 ml of buffered peptone and incubated at 37ºC overnight, an optical density index (ODI) of the sample was then determined at a wavelength of 540nm using a spectrophotometer. The sample was diluted using buffered peptone water until an ODI of 0.5 was obtained. A 10-fold series dilution of the samples was made and the dilutions were spread-plated on MAC and BCA, and incubated at 37ºC overnight, the colonies of the dilutions were counted and the cfu was confirmed. From the pilot studies done, 1 x 10^9 cfu's per ml was used. However after the first challenge the dose was reduced to 1 x 10^8 cfu's

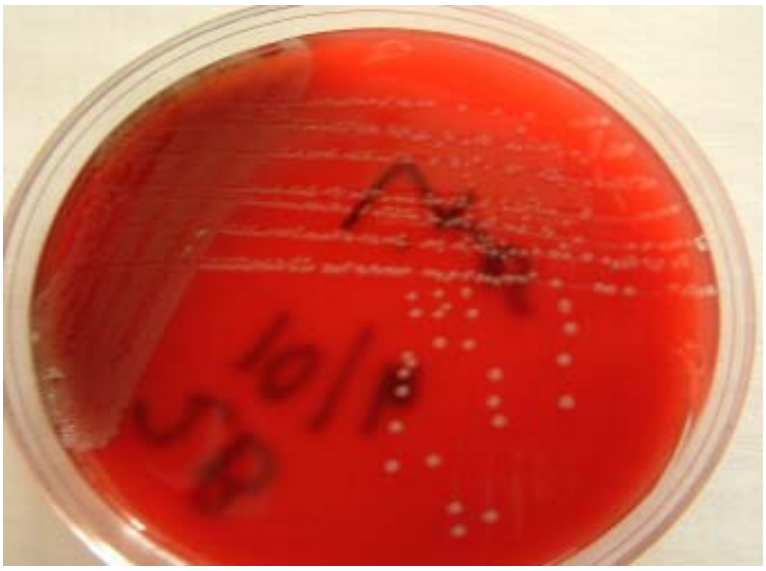
per ml in each case. The birds were then inoculated with 318/03 using a 1ml syringe directly down the oesophagus.

Culturing to determine SG colonisation **(for a full explanation refer to section 2.1.1)**

Faecal swabs, intestinal and crop scrapings

Faecal swabs, intestinal and crop scrapings were taken. Scrapings were done using a sterile pair of dissection scissors and the surface of the tissue was scraped off and placed in 10 ml of buffered peptone and shaken. They were incubated overnight at 37°C and then plated on MAC and BCA. These plates were incubated at 37°C overnight and checked for growth. If *Salmonella* was seen on the plates (grey round colonies on BTA and non-lactose fermenting colonies on MacConkey), a biochemical kit test (API 10S, Biomerieux, France) was performed to verify the presence of SG.

Figure 1: *Salmonella gallinarum* colonies collected from the duration of protection trial and plated on BTA.



Organ swabs and body/spleen weight ratios

Liver, lung and spleen swabs were taken under sterile conditions and plated directly onto MAC agar and BCA. The plates were incubated at 37°C overnight and checked for SG growth. A spleen to body weight percentage ratio was calculated, this was done by dividing the birds' spleen weight by its dead body weight and multiplying by 100.

Eggs

Eggs were washed with 70 % alcohol and allowed to dry in a sterile egg rack within a laminar flow cabinet (Gosair SA). They were then opened using sterile scissors and gloves and the contents placed in 100 ml buffered peptone, shaken, then incubated at 37°C overnight. 0,1 Millilitre alliquots were plated on MAC and BCA, and incubated overnight at 37°C. The rest of the procedure was done as for faecal swabs.

Serology

Blood was collected from the wing vein, allowed to clot and the serum was removed and tested using the BWD plate agglutination test. This is a universally accepted test whereby SP specific antigens were added to the serum sample. SG antibodies if present in the serum would bind to these antigens and agglutination would occur. Visible agglutination was taken as positive for SG and was scored as indicated in the key below (Intervet, 2001).

A key, for determining, the serological status of the serum samples using the BWD plate agglutination test (figure 2).

-: No clumps, no background clearing

+/-: Small clumps, mostly near the periphery, no background clearing, potentially a false positive.

+: Small clumps, no background clearing.

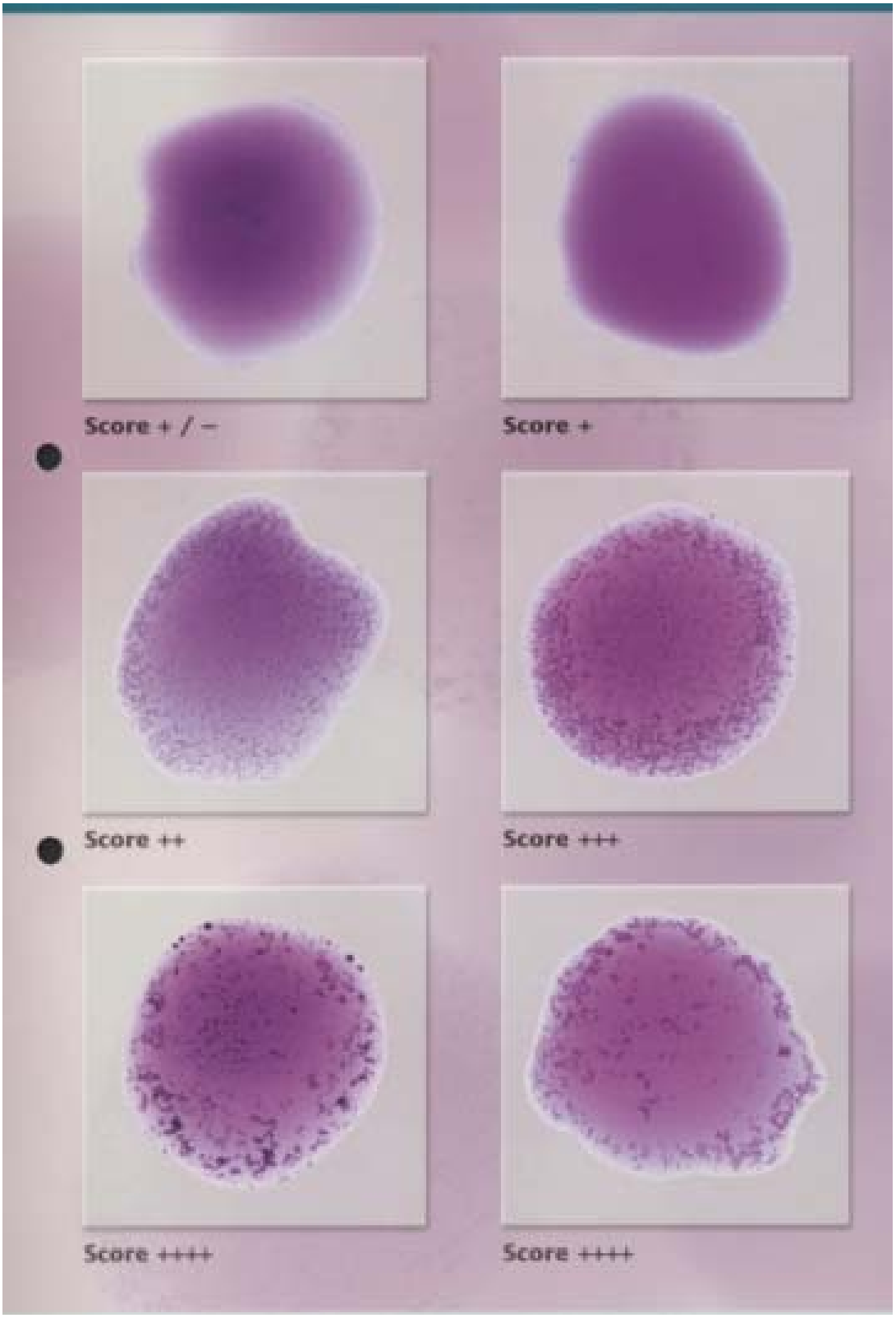
++: Medium sized clumps almost complete background clearing.

+++: Large clumps almost complete background clearing.

++++: Very large clumps, mostly in the periphery, complete background clearing.

The mean titres for Figure 4 were calculated by giving numerical values to the specific titres i.e. + = 1, ++ = 2 etc. and then calculating mean values for each group which were plotted on the graph.

Figure 2: Visual representation of the BWD serum plate agglutination test. (Intervet, 2001)



3.2.2 Routine Management Procedures

Birds

Unvaccinated day-old, commercial brown Hyline layers were purchased.

Feed

The birds were fed *ad libitum* throughout the trial. From day old for the first 6 weeks they were fed a commercial starter ration from Epol. The birds were then fed grower pellets (Epol) until 18 weeks of age. From 18 weeks onwards the birds were fed a commercial layer pellet ration (Epol).

Housing

On arrival at day-old the birds were housed in commercial rearing cages and were moved to commercial laying cages when they were split up into their groups at 40 days old.

Water supply

Water was supplied *ad libitum* throughout the trial. Initially in the rearing cages water was supplied in bell drinkers, once the birds were large enough to drink off the nipples the chick fonts were removed.

Test product

The OBPft vaccine was tested. The registration holder of the vaccine used in this trial is Onderstepoort Biological Products (OBP) (Ltd) Reg. No. G0107, (2000/0226 B6/06) (OBP product catalogue, 2004). The viability of the vaccine was not checked as the latest batch

was obtained on the day of vaccination and was certified by OBP to contain the required number of viable bacteria.

Vaccination

The vaccine was administered orally to one group and via intra-muscular injection to the other group. For both routes of vaccination the freeze-dried vaccine was reconstituted with the 100ml distilled water provided along with the vaccine. Each bird in the oral vaccine group was given 1 ml of the reconstituted vaccine directly into the oesophagus using an insulin syringe. Each bird in the intra-muscular vaccination group was injected into the pectoral muscle with 1ml of the reconstituted vaccine.

Vaccination schedule

The birds were vaccinated at 10 and 14 weeks of age.

Necropsy procedures

The necropsies were all performed in the same way by the same investigator. On opening the birds' abdominal and thoracic cavities, the liver was in full view with some intestines visible as well as the heart and lungs. Sterile pairs of scissors and forceps were used to collect organ specimens. Any visible pathology of the liver, spleen and lungs were recorded and separately swabbed for bacterial culture before being removed. The intestinal tract was then opened and observations made of lesions before mucosal scrapings were made of the caecal tonsils, Peyer's patches and crop for bacteriological examination. Once it was discovered that diseased birds had grossly enlarged spleens, it was decided to measure the spleen weight to body weight ratio of the rest of the birds. Before opening the birds their

body weight was measured, this weight was compared with the weight of the spleen that was removed during necropsy.

Euthanasia

Birds were euthanased by the intra-cardiac administration of 1ml of sodium pentobarbitone, (Eutha-nase, Centaur Bayer). All carcasses were securely bagged and incinerated at the Onderstepoort Veterinary Institute's incinerator.

3.2.3 Statistical analysis

For the duration of protection trial, the mean death times were taken to determine whether there was a significant difference between the two vaccinated groups and the control group in each of the challenge times (8, 16, 24 week challenges), but also to test whether there was a significant difference between the mean death times of the vaccinated groups (injected and oral). For this an F-test two-way ANOVA was performed, and $P < 0.05$ was considered significant (Statistical Analysis Software version 8.02) (Human, 2005). The statistical test was done with a confidence interval (CI) of 95 % ($\alpha = 0.05$).

The following variables were compared statistically:

- Group vaccinated parenterally with the control group
- Group vaccinated *per os* with the control group
- The injected group with the oral group
- Between the different times of challenge

The first test performed was to determine whether there was any significant difference between the injected and control group. Secondly, the same test was performed for the

orally vaccinated and control group. Thirdly the same test was performed between the two methods of vaccination (injected and oral). The fourth test was performed to test if there was any significant difference between the mean values obtained at the different challenge times (8, 16 and 24 week challenges).

In the ANOVA the death time in hours was taken to the nearest checkpoint in each group, post-challenge, where the challenge time was zero hours (Table 21). The surviving birds were those that did not die after challenge but were euthanased 14 days post SG challenges. They were allocated a death time in hours relating to their euthanasia fourteen days post challenge (336 hours). These birds could have lived for another year, or could have died the next day, this time of death allocation was only made to allow for closure of the experiment and to show the difference in death times of vaccinated versus control birds. The time to death for each bird was recorded three times a day, namely at 08:00; 14:00 and 16:30.

Egg production was monitored over the entire duration of protection trial. An ANOVA was performed using a 95 % CI comparing the two vaccinated groups and the control group. Then to confirm which groups were significantly different an unpaired T-test was performed using a 95 % CI.

No statistical work was done on the safety trial data due to the limited number of birds used, as only two birds per group were euthanased and tested per time frame. This did not give us enough data to do accurate statistical analysis.

CHAPTER 4

RESULTS

4.1 SAFETY TRIAL RESULTS

Figure 3: Splenomegally found in a bird seven days after vaccination.

In figure 3 the grossly enlarged spleen on the right was removed from a bird vaccinated parenterally (seven days post-vaccination), and the spleen on the left is taken from an unvaccinated control bird.

A brief reminder of the various groups involved. Group 1 was vaccinated at 10 and 14 weeks of age by intramuscular injection. Group 2 was vaccinated at 10 and 14 weeks of age by oral gavage. Birds in groups 3 and 4 received a single vaccination at 14 weeks by intramuscular injection or oral gavage respectively.

Tables 13 – 17 show the results pertaining to the different test days post-vaccination in the safety trial.

Table 13: shows that all the cultures were negative, and serology was inconclusive in the tests performed 2 days post-vaccination. The birds' spleens were enlarged and showed discolouration (Figure 3). This was a good indication of an early immune response to vaccination.

Seven days post vaccination, serology was still inconclusive, and SG was cultured from only one spleen from group 3. The spleens of all the birds were enlarged; some lungs showed signs of discolouration and peritonitis was found in two of the birds' body cavities (Table 14).

Fourteen days after vaccination group 1 showed a dramatic increase in serological titres, the other groups were still inconclusive. Only one culture was positive, this was a spleen culture from group 3. Pathologically the spleens were still enlarged but had decreased in size compared with the previous week's results, some spleens were still discoloured. After the results of the previous week spleen/body weight ratios were performed. The normal ratio is between 0.17% and 0.20% as taken by the values of the unvaccinated control birds in this trial and previous pilot studies (Table 15). The lungs of most of the birds were discoloured, showing the widespread effect of the vaccine.

Twenty-one days after vaccination the serological titres had increased in all the groups except group 4. The pathology was returning to normal, with a few

discoloured lungs, livers and spleens, the spleen weights were also decreasing. SG cultures from the spleens were positive in all the birds from groups 3 and 4 (Table 16).

By twenty-eight days post vaccination only group 1 had any significant serological response left, all the other groups' agglutinating antibodies had returned to inconclusive levels. All the cultures were negative, and the organ pathology had returned to normal in all but one speckled spleen. The spleen/body weight ratios had also decreased back to the normal range (Table 17).

Table 13: Necropsy results 2 days post-vaccination

Group	1		2		3		4	
Bird	1	2	1	2	1	2	1	2
Serology	+/-	+/-	+/-	+/-	+/-	+/-	neg	+/-
Necropsy scores (refer to page 40)	Lung 1 Spleen 2	Lung 1 Spleen 2	Spleen 2	Spleen 2	Spleen 2	Spleen 2	Spleen 2	Spleen 2
Cultures								
Liver swabs	neg	neg	neg	neg	neg	neg	neg	neg
Spleen swabs	neg	neg	neg	neg	neg	neg	neg	neg
Intestine/ crop scrapes	neg	neg	neg	neg	neg	neg	neg	neg
Faecal swabs	neg	neg	neg	neg	neg	neg	neg	neg

Table 14: Necropsy results 7 days post-vaccination

Group	1		2		3		4	
Bird	1	2	1	2	1	2	1	2
Serology	+	+/-	+/-	+/-	+/-	+/-	+	+/-
Necropsy scores	Spleen 3 Intestine 2	Spleen 3 Intestine 2	Spleen 3	Spleen 3	Spleen 3 Lung 1	Spleen 3 Lung 1	Spleen 3 Lung 1	Spleen 3 Lung 1
Cultures								
Liver swabs	neg	neg	neg	neg	neg	neg	neg	neg
Spleen swabs	neg	neg	neg	neg	**Pos**	neg	neg	neg
Intestine/ crop scrapes	neg	neg	neg	neg	neg	neg	neg	neg
Faecal swabs	neg	neg	neg	neg	neg	neg	neg	neg

Table 15: Necropsy results 14 days post-vaccination

Group	1		2		3		4		Neg. Control
Bird	1	2	1	2	1	2	1	2	
Serology	++	+++	+/-	+/-	+/-	+/-	+/-	+/-	neg
Necropsy scores	Lung 1	Lung 1	Spleen 1	Spleen1 Lung 1	Spleen 2 Lung 1	Spleen 1 Lung 1	Spleen 1 Lung 1	Spleen 1 Lung 1	-
Cultures									
Liver swabs	neg	neg	neg	neg	neg	neg	neg	neg	neg
Spleen swabs	neg	neg	neg	neg	**Pos**	neg	neg	neg	neg
Intestine/ crop scrapes	neg	neg	neg	neg	neg	neg	neg	neg	neg
Faecal swabs	neg	neg	neg	neg	neg	neg	neg	neg	neg
Spleen wt (g)	3.0	2.9	3.6	4.0	4.7	3.7	3.8	3.4	3.1
Body wt (g)	1644	1655	1743	1725	1597	1737	1643	1454	1590
% Spleen /body wt	0.18	0.17	0.20	0.23	0.29	0.21	0.23	0.23	0.19

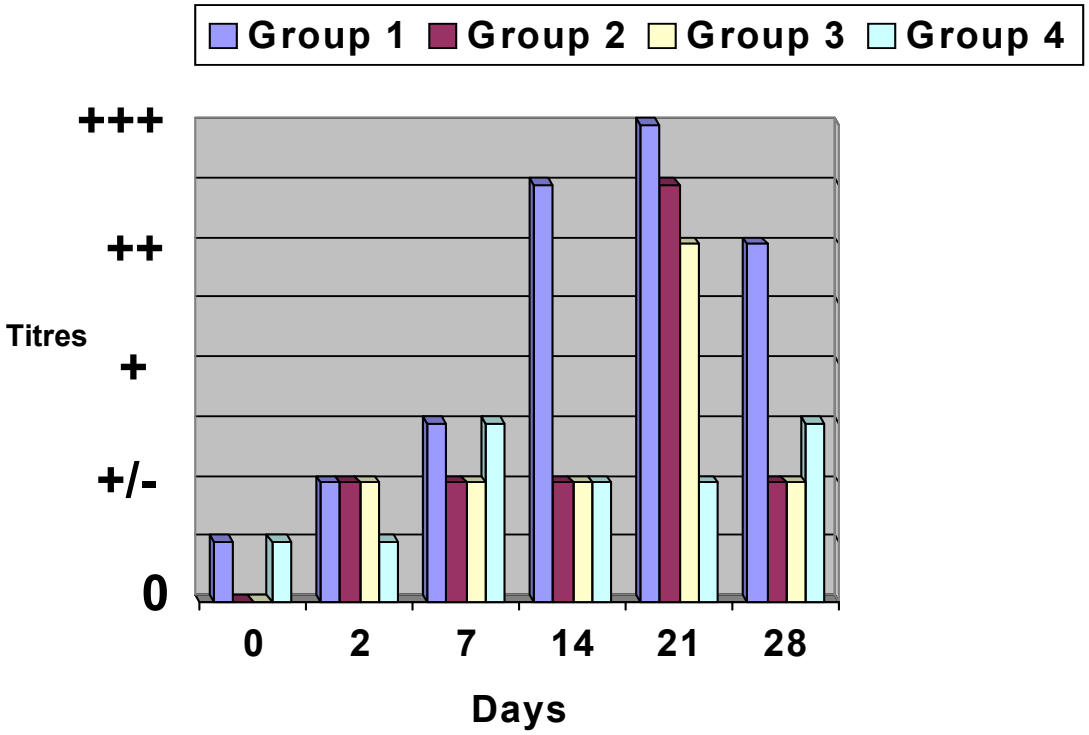
Table 16: Necropsy results 21 days post-vaccination.

Group	1		2		3		4		Neg. Control
Bird	1	2	1	2	1	2	1	2	
Serology	+++	+++	++	+++	++	++	+/-	+/-	neg
Necropsy scores	Lung 1 Liver 1	Lung 1	Spleen 1	-	Spleen 2	Spleen 1	Liver 1	-	-
Cultures									
Liver swabs	neg	neg	neg	neg	neg	neg	neg	neg	neg
Spleen swabs	neg	neg	neg	neg	**Pos**	**Pos**	**Pos**	**Pos**	neg
Intestine/ crop scrapes	neg	neg	neg	neg	neg	neg	neg	neg	neg
Faecal swabs	neg	neg	neg	neg	neg	neg	neg	neg	neg
Spleen wt (g)	3.5	3.6	4.5	2.7	4.0	3.9	3.0	3.2	3.1
Body wt (g)	1713	1759	1915	1489	1642	1721	1861	1678	1709
% Spleen /body wt	0.20	0.20	0.23	0.18	0.24	0.22	0.16	0.19	0.18

Table 17: Necropsy results 28 days post-vaccination

Group	1		2		3		4	
Bird	1	2	1	2	1	2	1	2
Serology	++	++	+/-	+/-	+/-	+/-	+/-	+
Necropsy scores	-	-	-	-	-	Spleen 1	-	-
Cultures								
Liver swabs	neg	neg	neg	neg	neg	neg	neg	neg
Spleen swabs	neg	neg	neg	neg	neg	neg	neg	neg
Intestine/ crop scrapes	neg	neg	neg	neg	neg	neg	neg	neg
Faecal swabs	neg	neg	neg	neg	neg	neg	neg	neg
Spleen wt (g)	2.9	3.1	3.1	3.3	3.2	4.0	3.4	3.1
Body wt (g)	1731	1733	1786	1816	1792	1836	1739	1768
% Spleen /body wt	0.16	0.18	0.17	0.18	0.18	0.22	0.19	0.17

Figure 4: Combined serological titres taken over the safety trial.



4.2 DURATION OF PROTECTION TRIAL RESULTS

4.2.1 Challenge 8 weeks post-vaccination: (22 weeks old)

Serology

Injected group:

Pre-challenge the titres of the birds were on average negative, 5 samples were negative and five samples were at the +/- level; by 7-days post-challenge all serum samples were positive. At 14-days post challenge the titres had increased further. (Fig 5)

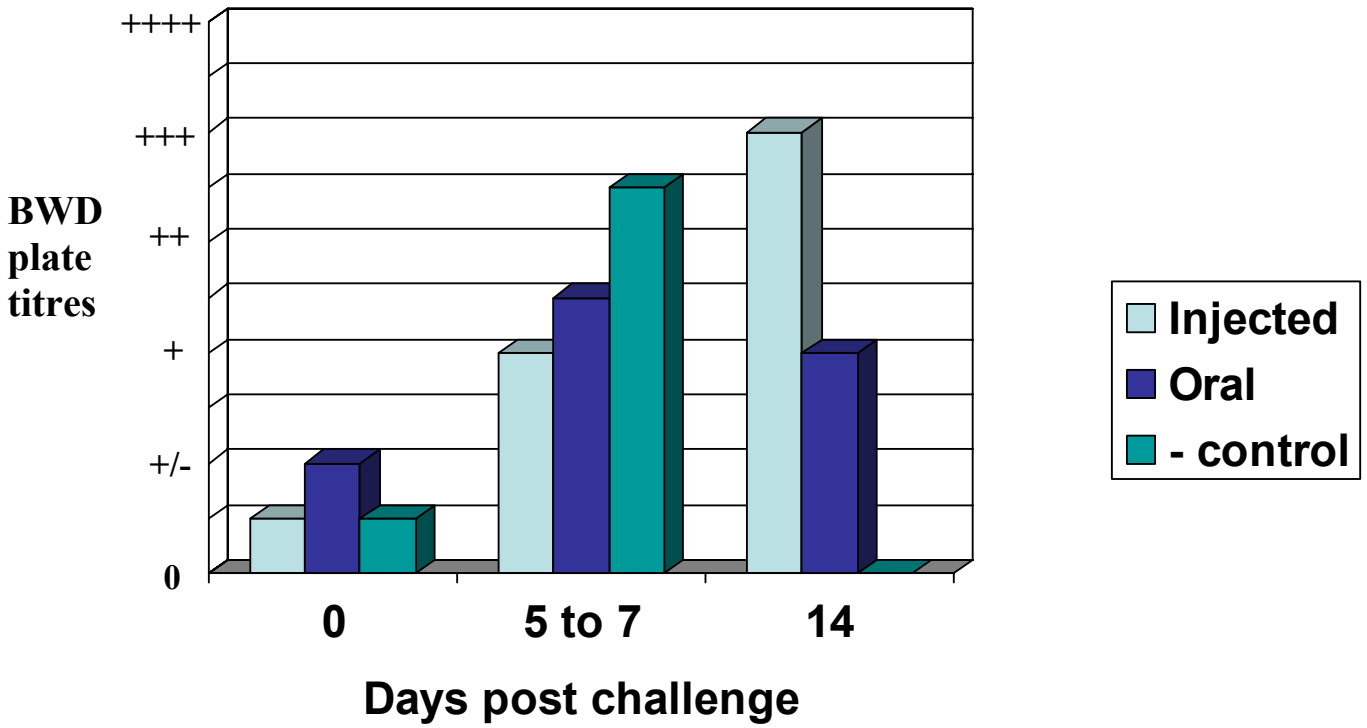
Orally vaccinated group:

Pre-challenge, the titres of 4 of the samples were above +/- value, giving the titres a positive status and 6 samples were below the +/- value hence considered negative. By 7 days post-challenge all serum titres had increased. At 14 days post-challenge the samples averaged fairly consistent with the previous week's results (Fig 5). The pre-challenge titres were slightly higher in this group than group 1, however not significantly so. They were still within the +/- rage which incorporates false positives, hence either cross-reactivity could have caused this or they were as a result of interpretive problems associated with the serological tests.

Unvaccinated control group:

Pre-challenge the titres were on average negative, with 6 samples negative and four samples at +/- level. From day's 5-7 post challenge, the samples had to be taken from the heart as the birds were dying acutely, all the samples taken were positive. By 14 days post-challenge all the controls were dead (Fig 5).

Figure 5: Mean serological titres taken after the challenge done 8 weeks post-vaccination



Necropsies

Injected group:

Two birds died as a result of SG infection 5 days post challenge. These birds' spleens and livers were enlarged and lungs were grey in colour. Cultures made of these organs were all positive for SG.

One bird in the injected group died 12 days post challenge (Table 18), this bird had a grossly enlarged spleen, the lungs were grey in colour and the liver was enlarged and bronze in colour. These organs were cultured and were positive for SG. The bird also had septicaemia and egg peritonitis due to *Escherichia. coli* infection.

The 7 birds' euthanased at the end of the trial period looked lively and healthy. At necropsy 6/7 birds' spleens, livers and lungs were normal. They did, however, show signs of secondary bacterial infections. One had egg peritonitis, and five had a mild airsacculitis. There was one bird in this group that had an enlarged spleen, liver and a grey lung from which SG was cultured. Furthermore *E. coli* was cultured from the abdominal cavity in this bird. The organs of the other surviving birds were all negative for SG.

Orally vaccinated group:

One bird died 5 days post-challenge and two birds died 6 days post-challenge. These birds' spleens were slightly enlarged; their livers brown and their lungs were an off white colour. Cultures made of these organs were all positive for SG.

One bird in this group died 12 days post-challenge (Table 18), this bird had an enlarged spleen, the lungs were white with black patches and the liver was enlarged with a golden brown colour. SG was cultured from these organs as well as the airsacs, the body cavity and duodenum.

The birds' euthanased at the end of the trial period looked lively and appeared healthy. The spleens and lungs of all the birds were normal and, except for two that had enlarged livers, all the livers appeared normal. One bird had egg peritonitis due to a secondary *E. coli* infection. All the organs mentioned above were cultured and were negative for SG colonisation.

Unvaccinated control group:

Within 6 days post-challenge all the control birds were dead, seven died 5 days post-challenge and 3 died on the 6^{th} day (Table18). The spleens of these birds were moderately enlarged. The livers were enlarged, spotted and were golden brown in colour. The lungs were a creamy white colour. All the spleen and liver cultures were positive for SG colonisation. There were no secondary infections present.

Mean death times were calculated and used for statistical analysis section 4.3 Table 22.

Table 18: Mortalities in challenge done 8-weeks post-vaccination

Vaccination method	Mortalities	% protection	Challenge dose (cfu)
Injected group	3/10	70	1×10^9
Oral group	4/10	60	1×10^9
Neg. control group (Figure 12)	10/10	0	1×10^9

Figure 6: An enlarged liver of a control bird that died from FT infection.

Figure 7: An enlarged spleen from a control bird 6 days post-challenge.

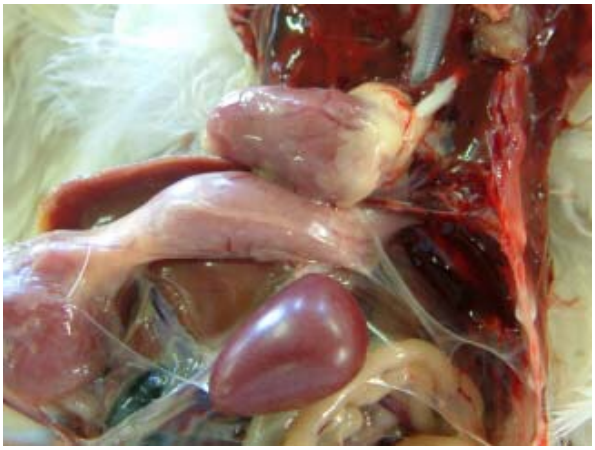
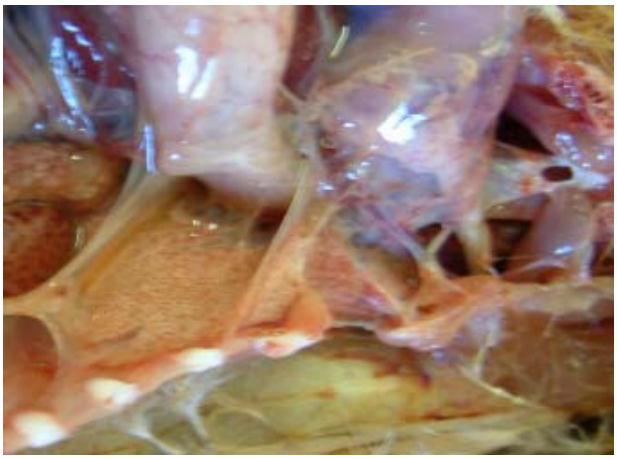


Figure 8: A pale lung from a control bird that died due to FT infection.



4.2.2 Challenge 16 weeks post-vaccination: (30 weeks old)

Serology

Injected group:

Pre-challenge, the titres of the 10 birds were as follows; seven were negative and three were at the +/- level; by 7-days post challenge all serum samples were positive for SG. At 14-days post challenge the titres were greatly increased. (Fig 9)

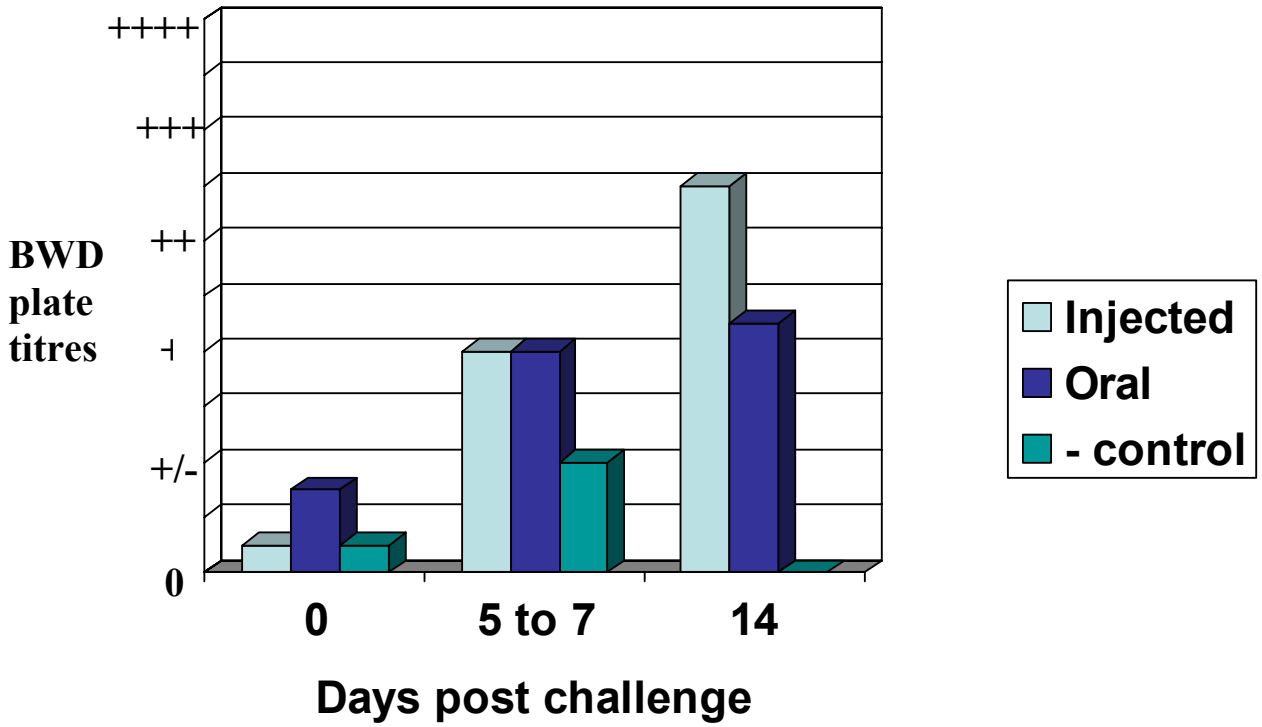
Orally vaccinated group:

Pre-challenge the titres of the 10 birds were as follows; four samples were negative, five samples were at +/- level and one sample was at the + level. By 7 days post-challenge the samples were positive. At 14 days post challenge the samples were positive and fairly consistent with the previous week's tests. (Fig 9)

Unvaccinated control group:

Pre-challenge, the titres of the 10 birds as follows; seven samples were negative and three samples were at the +/- level. At 5-7 days post challenge the samples were all at the +/- level. By 14 days post challenge all the controls were dead. (Fig 9)

Figure 9: Mean serological titres taken after the challenge done 16 weeks post-vaccination



Necropsies

Injected group:

One bird died and one was euthanased on the 8th day post-challenge. The bird that was euthanased was dehydrated and lying on the floor of the cage with its eyes closed, it didn't have the strength to eat or drink. These birds' spleens and livers were enlarged and their lungs were pale in colour. Cultures made from these organs were all positive for SG colonisation.

One bird in this group died 10 days post-challenge (Table 19), this bird had an enlarged spleen, the lungs were white and liver was grossly enlarged and yellow in colour. These organs were cultured and were positive for SG. The bird also had egg peritonitis that was cultured positive for *E. coli*.

The 7 birds' euthanased at the end of the trial period looked lively and healthy. On inspection, all the birds' spleens and lungs were normal. Three birds had enlarged livers. Secondary infections occurred in three birds, two had egg peritonitis and one had a colisepticaemia. All the organs mentioned above were cultured, only two livers were positive for SG, all the other organs were negative for SG colonisation.

Orally vaccinated group:

Two birds died on the 8th day post challenge. These birds' spleens and livers were slightly enlarged, and lungs were a pale pink colour. Cultures made of these organs were all positive for SG colonisation.

One bird in this group died 9 days post-challenge (Table 19), this bird had an enlarged spleen and liver, the lungs were a cream colour. These organs were cultured and were positive for SG. There was a mild airsacculitis present.

The birds' euthanased at the end of the trial period looked healthy. At necropsy, 6/7 of the birds' spleens and lungs were normal, and except for two with enlarged livers the rest were normal. One bird had an enlarged spleen, with badly affected liver and lungs; this bird also had egg peritonitis. Another bird also had a mild airsacculitis. All the organs mentioned above were cultured, only two livers and one spleen was positive for SG colonisation.

Unvaccinated control group:

On the 7th day post challenge one of the control birds died and one was euthanased. The spleens of these birds were slightly enlarged. The livers were enlarged, spotted and were golden brown in colour. The lungs were a creamy white colour. All the spleen and liver cultures were positive for SG colonisation. There were no secondary infections present.

Seven birds died on the 8th day post-challenge. All of the livers were enlarged and a golden brown colour and splenomegally was found in five birds. All the lungs were a creamy white colour with black patches. All the organs above were cultured and were positive for SG colonisation. There were no secondary infections present.

The last control bird died on the 9th day (Table 19) and had an enlarged spleen, and badly affected liver and lungs. Cultures were made of the organs and all were positive for SG.

Table 19: Mortalities in challenge done 16-weeks post-vaccination

Vaccination method	Mortalities	% protection	Challenge dose (cfu)
Injected group	3/10	70	1×10^8
Oral group	3/10	70	1×10^8
Neg. control group	10/10	0	1×10^8

Graph of % protection (Fig 12)

4.2.3 Challenge 24 weeks post-vaccination: (38 weeks old)

Serology

Injected group:

Pre-challenge the titres of the 10 birds, seven birds were negative and three were at the +/- level. By 7 days post-challenge all serum samples were at the +/- level. At 14 days post challenge the titres of the remaining birds had increased dramatically. (Fig 10)

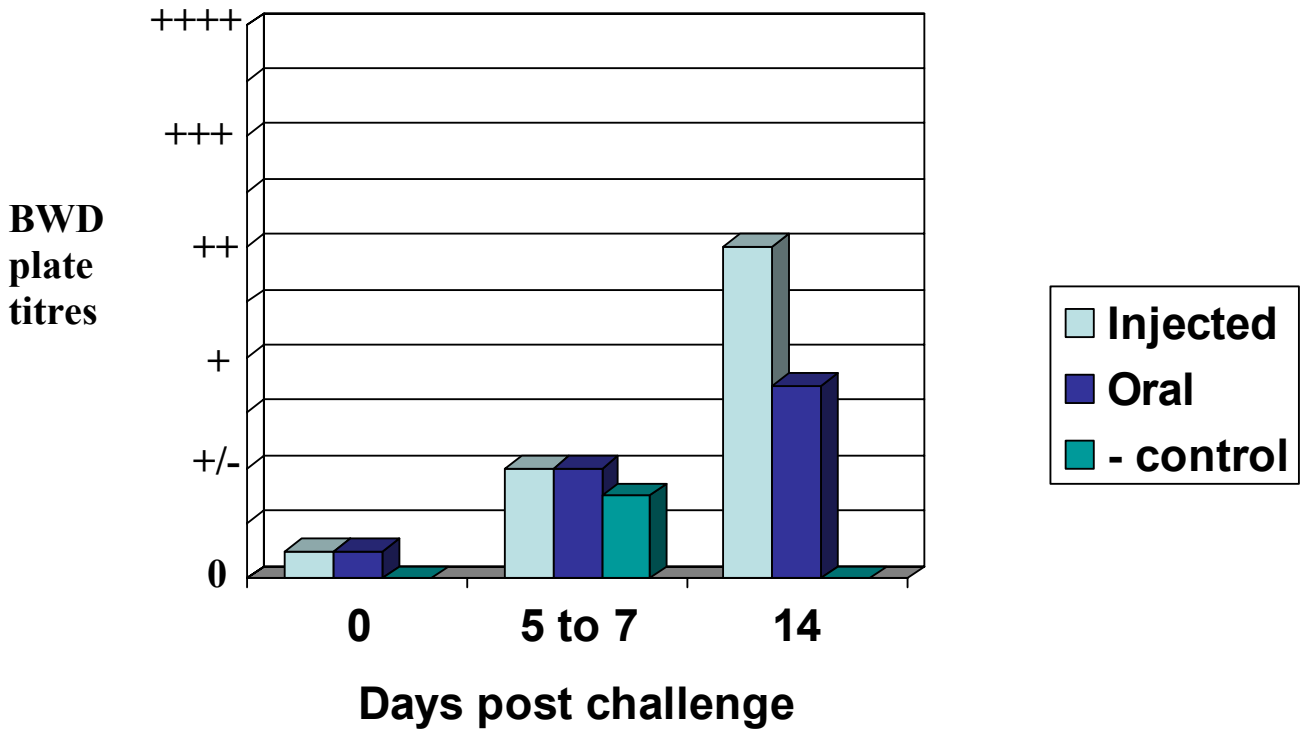
Orally vaccinated group:

Pre-challenge the titres of the 10 birds, seven birds were negative and three were at the +/- level. By 7 days post-challenge the samples were at the +/- level. At 14 days post-challenge the samples showed a higher titre. (Fig 10)

Unvaccinated control group:

Pre-challenge the titres of all 10 birds were negative. At 7-days post challenge the samples remaining had very low titres. By 14 days post-challenge all the controls were dead. (Fig 10)

Figure 10: Mean serological titres taken after the challenge done 24 weeks post-vaccination



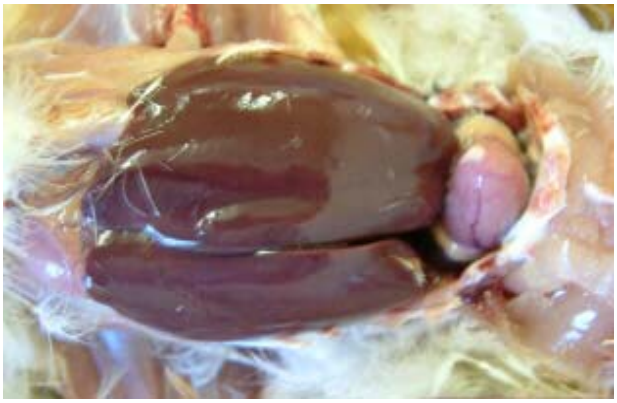
Necropsies

Injected group:

Seven birds died within 8 days post-challenge; one bird died 5 days, four died 6 days and two died 7 days post-challenge (Table 20). These birds' spleens and livers were enlarged, and their lungs were white in colour. Cultures made of these organs were all positive for SG colonisation, and three birds had egg peritonitis. One bird in this group died 11 days post-challenge, this bird had an enlarged spleen and liver, and the lungs were white in colour. These organs were cultured and were positive for SG. This bird also had egg peritonitis.

The two birds' euthanased at the end of the trial period looked lively and appeared healthy. At necropsy, both spleens and lungs were normal. One bird had an enlarged liver. There were no signs of secondary infection. All the organs mentioned above were cultured, one liver and spleen culture was positive for SG colonisation.

Figure 11: An enlarged liver from a bird in the injected group that died of FT infection



Orally vaccinated group:

Seven birds died within 8 days post-challenge; one bird died 5 days, five died 6 days and one died 7 days post-challenge (Table 20). These birds' spleens were slightly enlarged, their livers were enlarged and brown in colour, and their lungs were white with black specks. Cultures made of these organs were all positive for SG colonisation, and three birds showed signs of egg peritonitis.

The birds' euthanased at the end of the trial period looked healthy. At necropsy, the spleens and lungs were normal and one liver was brown in colour. There were no signs of secondary infection and all the cultures taken were negative for SG colonisation.

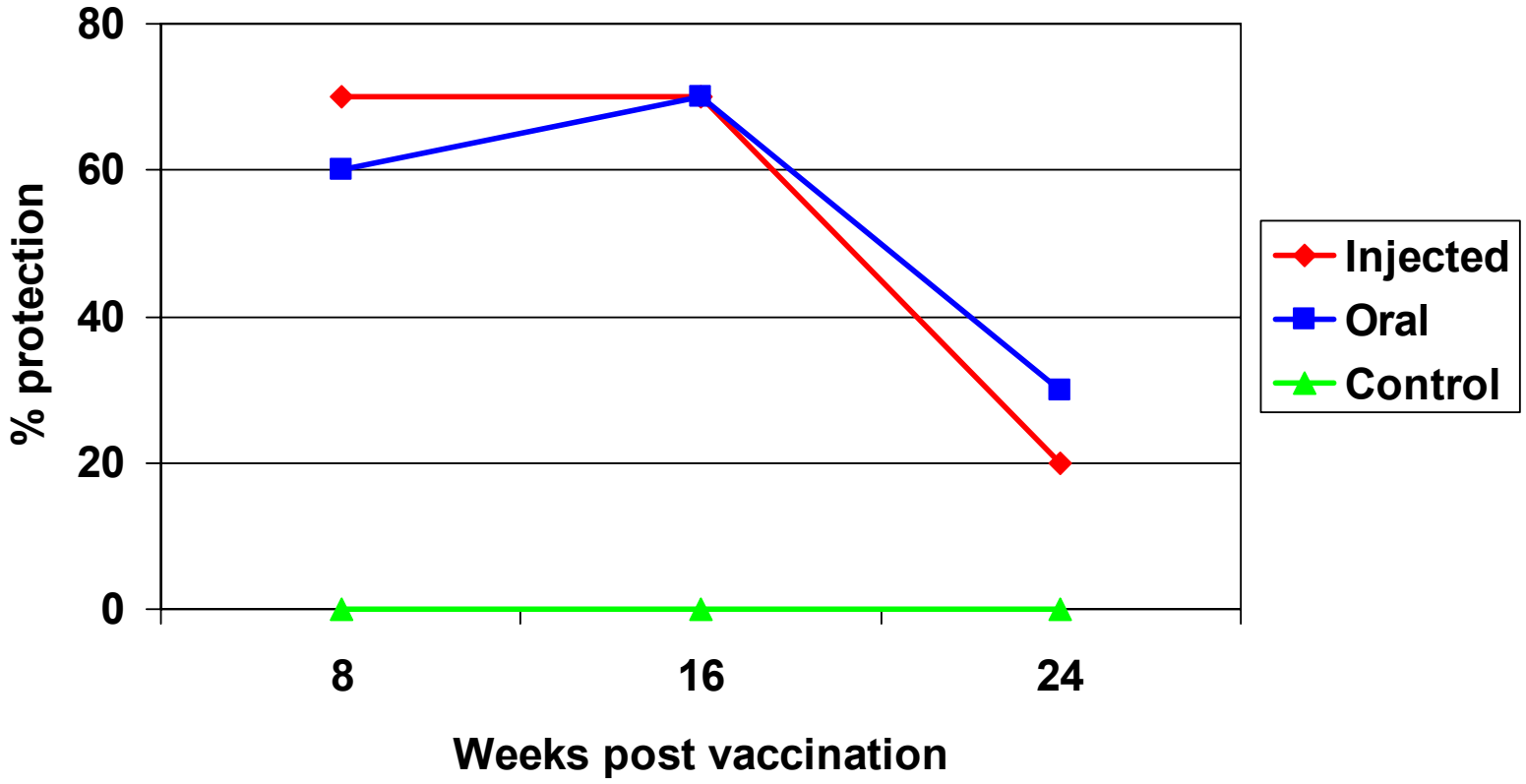
Unvaccinated control group:

All the control birds had died by the 8th day post challenge, five birds died 5 days and five died 6 days post challenge (Table 20). The spleens of these birds were slightly enlarged. The livers were enlarged, spotted and were golden brown in colour. The lungs were a creamy white colour. All the organs above were cultured and were positive for SG colonisation. There were no secondary infections present.

Table 20: Mortalities in challenge done 24-weeks post vaccination

Vaccination method	Mortalities	% protection	Challenge dose (cfu)
Injected group	8/10	20	1 x 10^8
Oral group	7/10	30	1 x 10^8
Neg. control group (Figure 12)	10/10	0	1 x 10^8

Figure 12: Percentage protection achieved against fowl typhoid mortality throughout the trial (Table 18, 19 and 20).



4.2.4 Egg production figures over the challenge trial

Egg production drops during the SG challenges are shown in Figure 13 and Table 21.

The number of eggs collected from all the challenges was combined for each vaccination group and the unvaccinated control group and compared to the number of birds available to lay. A percentage egg production was calculated each day (hen day egg production).

Figure 13: Percentage egg production change over the challenge trial

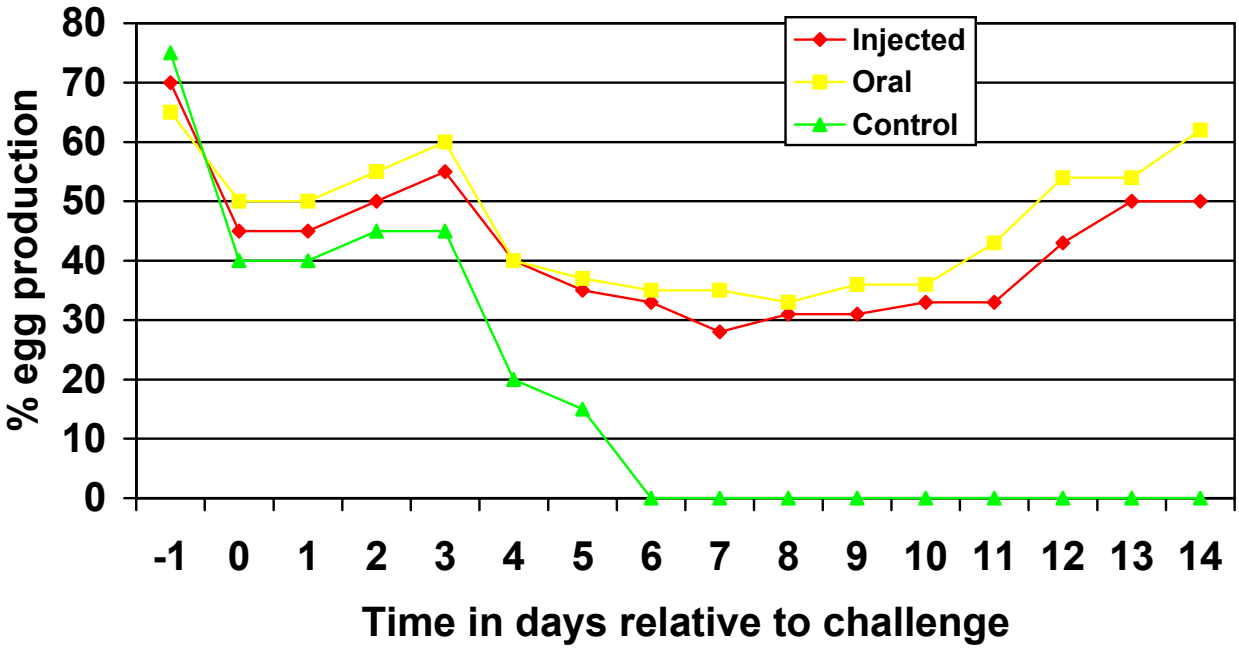


Table 21: Number of eggs collected during challenge trials with statistical values

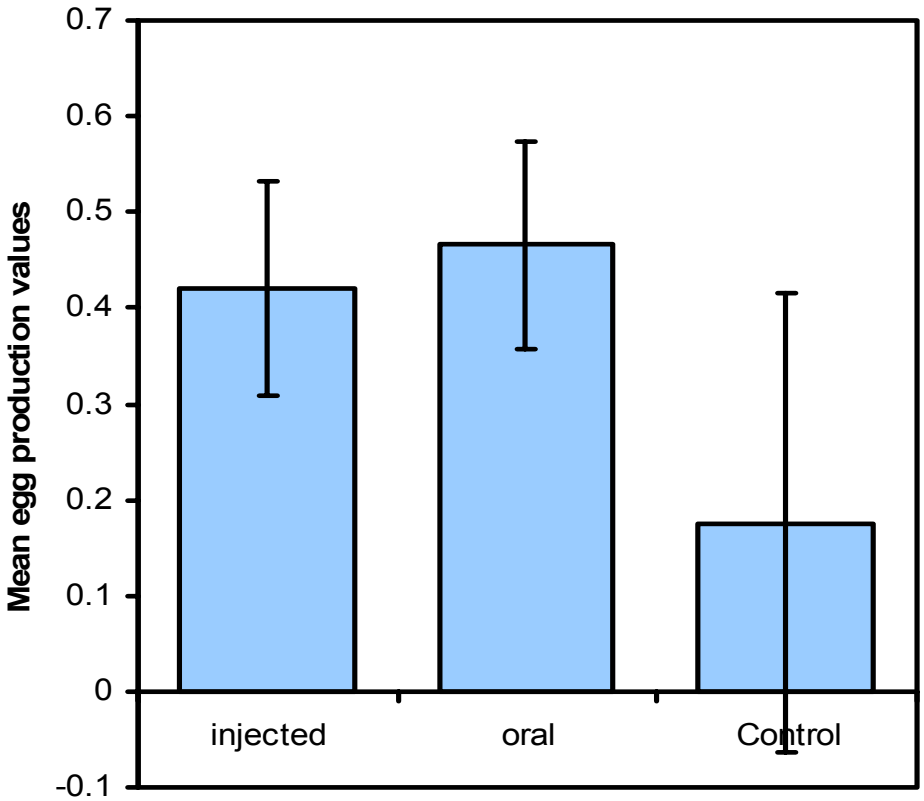
Days post challenge	Number of eggs collected/ number of birds		
	Injected	Oral	Unvaccinated Control
-1	14/20=70%	13/20=65%	15/20=75%
0	9/20=45%	10/20=50%	8/20=40%
1	9/20=45%	10/20=50%	8/20=40%
2	10/20=50%	11/20=55%	9/20=45%
3	11/20=55%	12/20=60%	9/20=45%
4	8/20=40%	8/20=40%	4/20=20%
5	7/20=35%	7/19=37%	2/13=15%
6	6/18=33%	6/17=35%	0/10
7	5/18=28%	6/17=35%	0/8
8	5/16=31%	5/15=33%	0/1
9	5/16=31%	5/14=36%	0/0
10	5/15=33%	5/14=36%	0/0
11	5/15=33%	6/14=43%	0/0
12	6/14=43%	7/13=54%	0/0
13	7/14=50%	7/13=54%	0/0
14	7/14=50%	8/13=62%	0/0
Mean	0.42	0.466	0.175
Std Deviation	0.1125	0.109	0.239
95% CI	0.055	0.0535	0.117
P value (ANOVA)			$1.54x10^{-5}$

Statistically an ANOVA and unpaired T-test were performed.

ANOVA

This test compared the three groups seen in Table 21. Firstly, all the data points were used, from day -1 to 14 (Fig 14). This gave a P value of 0.0000154 which is less than 0.01, showing that there was a significant difference between at least two of the three groups. This test was also performed using days 1 to 10 (Table 21). Here the P value was 0.000641, which again is less than 0.01, showing that there was a significant difference between at least two of the groups.

Figure 14: Mean egg production values with standard deviations over the challenge trial



Unpaired T-test

Results of the unpaired t-test indicate that there was a statistically significant difference in mean egg production between both vaccinated groups and the unvaccinated control group at the 1 % CI. There was no statistically significant difference in mean egg production between the two vaccinated groups.

4.3 COMPARISON OF MEAN DEATH TIMES

Table 22 shows the individual death times for each bird taken during the challenges. These results are in hours, worked out from zero hours at the time of challenge to the time of death.

Table 22: Death times of individual birds after challenge.

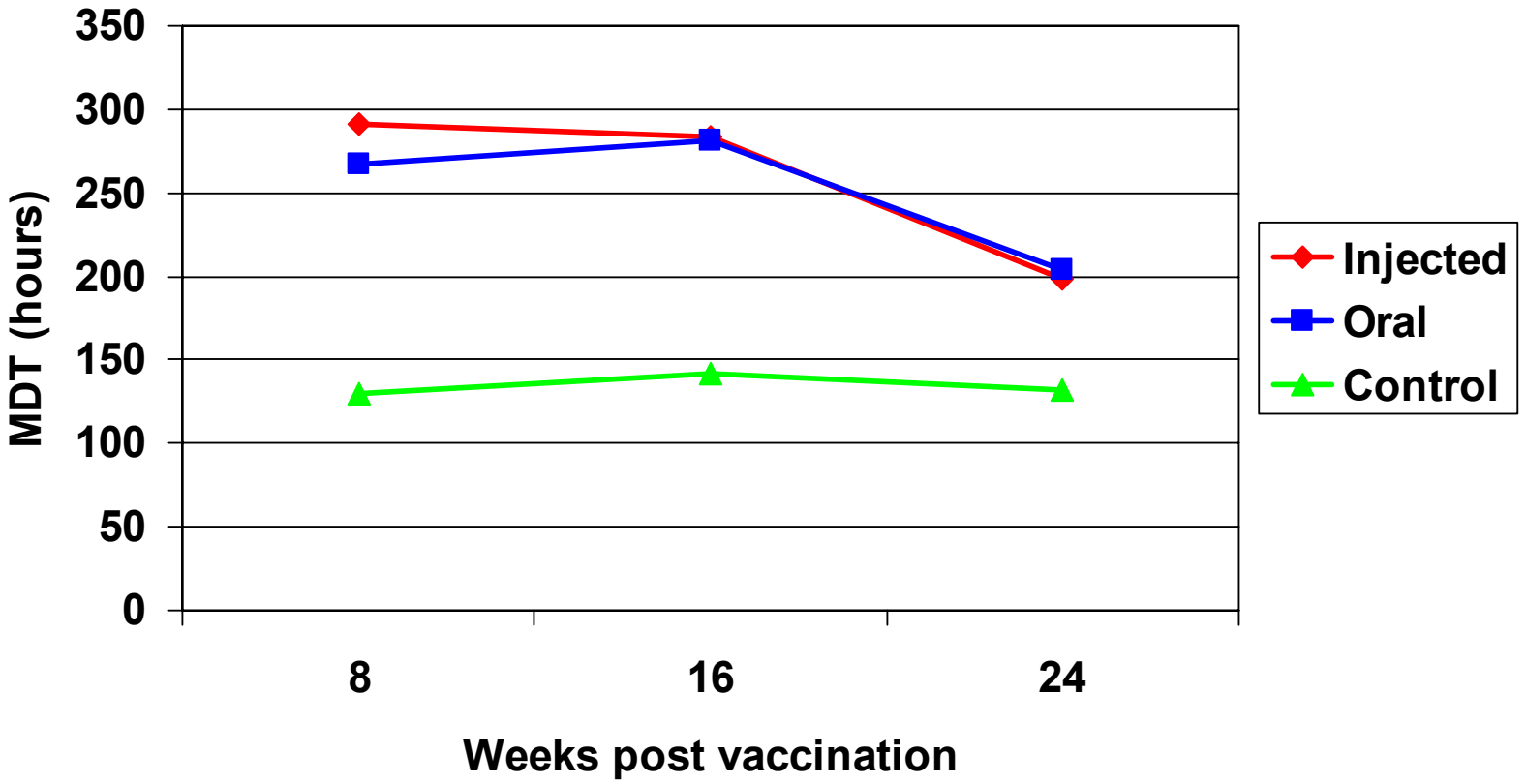
Treatment	22 week-old challenge (hr)	30 week-old challenge (hr)	38 week-old challenge (hr)
Injected	126, 144, 288, 336, 336, 336, 336, 336, 336, 336	144, 144, 198, 336, 336, 336, 336, 336, 336, 336	120, 144, 150, 150, 150, 168, 168, 264, 336, 336
Oral	120, 144, 144, 246, 336, 336, 336, 336, 336, 336	144, 144, 174, 336, 336, 336, 336, 336, 336, 336	120, 144, 144, 150, 150, 150, 168, 336, 336, 336
Control	120, 120, 120, 120, 126, 126, 126, 144, 144, 150	120, 120, 144, 144, 144, 144, 144, 144, 144, 168	120, 120, 120, 120, 120, 144, 144, 144, 144, 144

Using the data from Table 22 the MDT was calculated for each group and is in Table 23 and Figure 15. The MDT is shown as time in hours post challenge.

Table 23: Mean death times (MDT) of the duration of protection results

	22 week challenge	30 week challenge	38 week challenge	Treatment mean
Injected	291.0	283.8	198.6	257.8
Oral	267.0	281.4	203.4	250.6
Control	129.6	141.6	132.0	134.4
Time-frame mean	229.2	235.6	178.0	

Figure 15: MDT of all birds in the different groups taken over the entire, duration of protection trial.



4.3.1 Injected versus control groups

In Table 24 the injected group was compared to the control group and the relevant data was taken from Table 22 to work out the Sum of Squares (SS) and Mean Square (MS) to get an F-value (This is a number taken from an F-table set up for this test). It tested whether there was a significant difference between the MDT of the injected and control groups. From the data in Table 23 the F-ratio equalled 7.028 and was greater than the F-table result of 6.94 at a 95% CI. Thus there is a significant difference between the MDT of the injected and control groups over all the challenges.

Table 24: Injected group versus control group, statistics

Source	SS	MS	F-ratio	F-test-table
Treatment	4333.23	2166.62	MS_{time}/MS_{treat}	$F_{(2,4)0.05}$
Time-frame	15227.56	15227.56	= 7.028	= 6.94
Within	12544.07	6272.035		

4.3.2 Oral versus control groups

In Table 25 the oral group was compared with the control group and the relevant data was from Table 23. From the data in the last two columns of Table 25 the F-ratio equals 8.91 and this value is greater than the F-table result of 6.94 at the 95% confidence interval. Thus there was a significant difference between the MDT of the oral and control groups over all the challenges.

Table 25: Oral group versus control group, statistics

Source	SS	MS	F-ratio	F-test-table
Treatment	3029.04	1514.52	MS_{time}/MS_{treat}	$F_{(2,4)0.05}$
Time-frame	13502.44	13502.44	= 8.91	= 6.94
Within	4248.26	2124.13		

4.3.3 Injected versus oral groups

In Table 26 the injected group is compared with the oral group and the relevant data needed is taken from Table 23. From the data in the last two columns of Table 26 the F-ratio equals 0.008 and this value is much less than the F-table value of 6.94 with a 95 % CI. Thus there is no significant difference between the MDT of the injected and oral groups.

Table 26: Injected group versus oral group, statistics

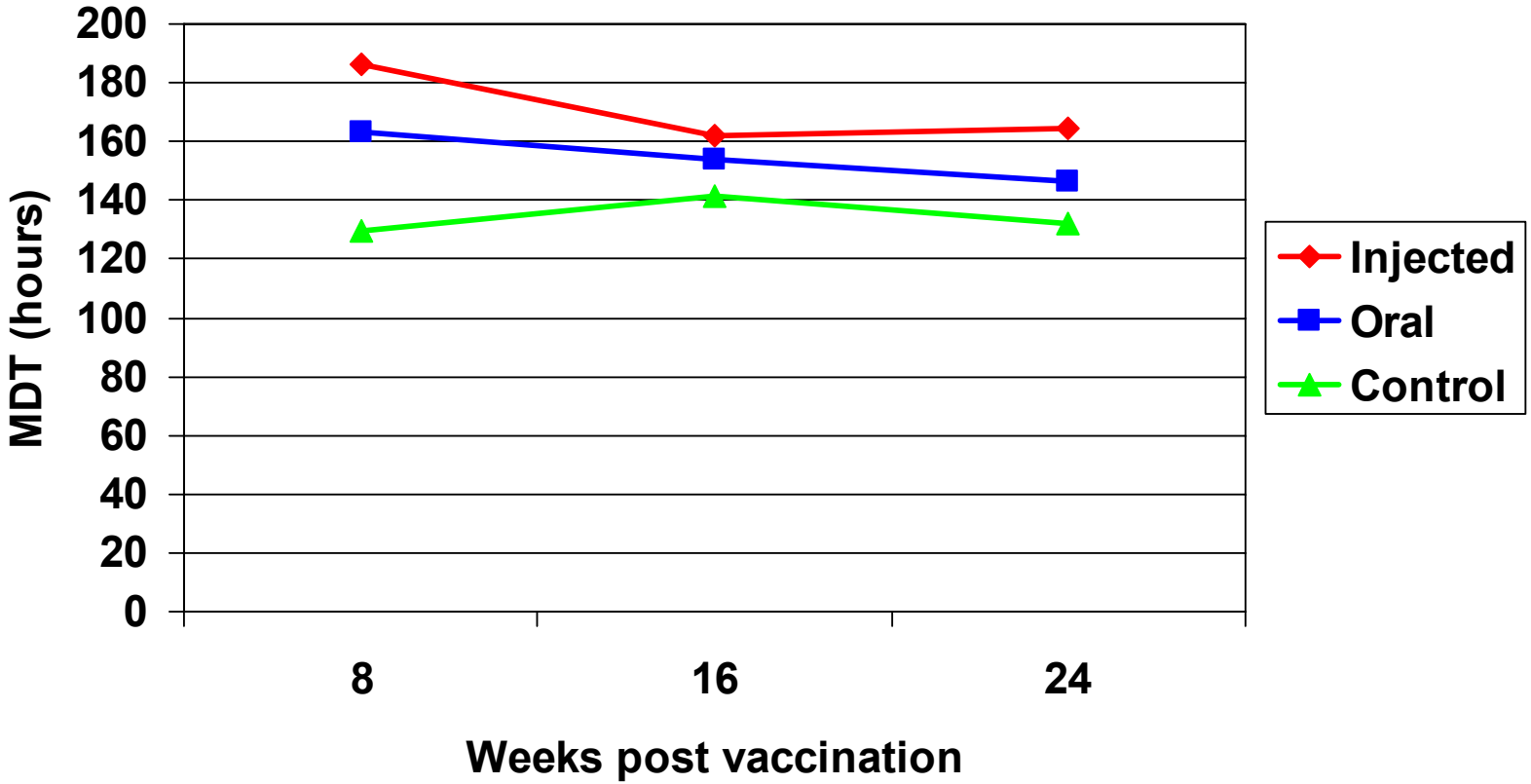
Source	SS	MS	F-ratio	F-test-table
Treatment	12755.52	6377.76	MS_{time}/MS_{treat}	$F_{(2,4)0.05}$
Time-frame	51.84	51.84	= 0.008	= 6.94
Within	2417.28	1208.64		

In the mean death times used, these death times were from the control groups over the three challenge dates post final vaccination.

4.3.4 Differences between the three challenges

Figure 16 showed the trend of MDT over the three challenges of only those birds that died directly due to FT infection. These values are not significantly different from each other over the three challenges. The data from the vaccinated groups was added to the graph (Fig. 16) as a comparison with no significant difference.

Figure 16: MDT in hours of only the birds that died directly due to FT infection, over the three challenge dates



CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 SAFETY TRIAL

5.1.2 Serological response

The antibody titres rose earliest, at 7 days post vaccination, and sustained themselves the longest in the group vaccinated twice by injection (Group 1).

The weakest serological response was seen in group 3. Group 4 also had a weak serological response. SG was isolated from the spleen of birds in group 3 and 4, indicating that they had not built up an immune response that was able to clear the vaccine strain.

Birds vaccinated orally tended to show a weaker and later serological response than birds vaccinated by injection, the response was also shorter-lived. This weak response does not necessarily mean that oral vaccination provided inferior immunity to that of injection. The protective immune response does not only consist of a humoral component, but also local mucosal and cellular components. Since SG is a facultative intracellular bacterium it induces a strong cellular immunity that is measurable by other means (Collins, 1974; Silva *et al*., 1981a; Wigley, Hulme, Powers, Beal, Berchieri, Smith and Barrow, 2005a). This is similar to the conclusion made by Cameron, Brett and Fuls, in (1974); who said that immunity is not dependant on the humoral response but rather more dependant on the cellular immune response. Certain tests may measure a

particular type of antibody, in this case agglutinating antibody, and thus does not reflect the entire protective response, it just indicates exposure.

5.1.2 Necropsy

Despite the fact that the vaccine did not cause any noticeable clinical signs, mild and transient pathology was noted in the liver, lung and spleen, between 2 and 14-days post vaccination. At two days post vaccination, the spleens of vaccinated birds were enlarged in comparison to unvaccinated birds. Spleen size was found to be a good indicator of an acute immune response to vaccination as splenomegally was observed 2 days post-vaccination and peaked 7 days post-vaccination (Tables 13 and 14). This was, however, short-lived and all spleens had returned to normal by 28 days post-vaccination.

5.1.3 Bacterial Culture

SG was only cultured from the spleens of birds that were immunized once at 14 weeks old, by both the oral and injected routes. This could be explained by the fact the birds that had been previously vaccinated, at 10 weeks old, were already primed, and were able to rapidly clear the vaccine strain. (Allan and Duffus, 1971; Saif, 2003)

SG could not be isolated from the faeces, throughout the trial, indicating that the vaccine is unlikely to be excreted and circulate in a population. The vaccine strain was also not isolated from the eggs. The shedding of live vaccines is a risk due to

the potential mutation of the vaccine strain into a pathogenic strain. Another problem associated with shedding of vaccines is the potential carrier status of adult birds, and the isolation of the vaccine strain in birds that may lead to a misdiagnosis of SG, this however has never been confirmed (Saif, 2003).

5.1.4 Controls

A small number of birds were used as negative controls in the safety trial. This was due to a few more birds dying than expected in the rearing process. In order not to remove birds from the rest of the trial, and knowing the length of the second trial and the possibility of losing more birds, the control group in the safety trial had to compensate for the losses. The vaccine strain of SG did not infect the negative control birds kept in cages alongside the vaccinated birds. These control birds remained healthy, had no gross pathology and did not seroconvert. This confirms the inability of the vaccine strain to spread to in-contact birds.

5.1.5 Safety (Conclusion)

The results indicate that the oral application of OBPft vaccine is as safe as the injected method when vaccinated at 10 and 14 weeks of age in commercial layer pullets. There were no clinical signs of disease or mortality, nor could any shedding of the vaccine strain be detected during the monitoring period of the safety trial. This data is similar to the findings by Young, Lee and Kang, (2005); they tested the safety of the SG9R vaccine when administered via injection.

One would expect some of the original vaccine dose to pass through the birds, perhaps an earlier test 24 hours post challenge of the faeces by culture as well as PCR may have shown this.

5.2 DURATION OF PROTECTION TRIAL

5.2.1 Validity of challenge model

Clinical illness associated with the bacterial challenge was noticed throughout the challenge trial in non-immunised birds. These challenged birds were listless, and had liquid slimy diarrhoea. This diarrhoea was often a bright yellow/green colour. Noticeably at about 4 days post-challenge the control birds began to stop eating. The 318/03 SG challenge strain used in this trial is highly virulent. This was shown by the 100 % mortality in all the control groups in this trial. This was higher than what was obtained with the younger birds in the second pilot study using a lower dose. This indicates that the stresses of lay could decrease the birds' resistance to SG challenge.

5.2.2 Serological response

There was an increase in antibody titre in all the vaccinated challenge groups by seven days post-challenge, while most of the unvaccinated control groups died too acutely for a measurable response. The antibody levels in the vaccinated groups increased further in the test done 14 days post-challenge. In the duration of protection trial, the injected group had a stronger serological response than the group vaccinated orally. This variation in serological response between the vaccinated groups might be attributed to less of the vaccine strain able to invade the organs from the intestines in birds vaccinated orally.

Salmonella cause a brisk local and systemic inflammatory response associated with the T helper cell and antibody responses of the IgG and IgA classes (Collins, 1974; Janeway *et al*., 2001). The IgM molecule is the first to be produced when a foreign organism is recognised and has a better ability to agglutinate antigen than IgG, as it has 10 binding sites. Our serological tests aimed to detect the increased levels of IgM in the blood. Once the initial reaction has taken place the IgM molecules are replaced by IgG molecules which agglutinate antigens less effectively than IgM. The oral administration of the vaccine is more likely to elicit a local mucosal immunity that may well be protective but won't reflect well in the agglutination test, and this can be seen by the lower serological response (Figures 5, 9 and 10) with the same protection offered.

The serological responses after the first two challenges (Figures 5 and 9) were very similar. The immune response in the last challenge (Figure 10) took a whole week longer to register on the tests. One of the reasons was due to the high percentage of acute mortalities in these groups, and skewing of the data due to decreased sample size. The serological responses were transient in all groups; this is probably due to the nature of the test used and that humoral response is short-lived. The vaccine strain may not be pathogenic enough to enter the circulation in the second vaccination hence the response offered would be primarily local. Considering the similar protection offered by the two routes of vaccination, this suggests that the strength of the sero-conversion is not a good indicator of the protection offered.

5.2.3 Protection versus Mortality

Mortalities due to SG were expected to start at 5 – 7 days post-challenge. This was something noted in the pilot studies prior to this trial. However, in the first challenge done 8 weeks post-vaccination all the control birds died peracutely by the 6th day post-challenge, without clinical signs. Another problem was that there were also mortalities in the vaccinated groups. The injected group had three mortalities, two of which died acutely within 6 days post-challenge. The oral group had four mortalities, three of which died within 6 days post-challenge. This was unexpected and showed that even vaccinated birds could be fatally susceptible to infection with SG if the strain is highly virulent and/or the infectious dose is high enough. The protection offered by the vaccine whether given orally or by injection gave good protection compared with the control group. This was different to the pilot study where 1 x 10^9 cfu's per dose was found to induce the expected mortality pattern. This dose is much higher than the challenge dose expected in natural infections, which is thought to be cumulative (Audisio and Terzolo, 2002; Silva *et al*., 1981a; Saif, 2003)

The reason for the sudden change in susceptibility to the SG challenge strain is thought to be due to the age of the birds in this challenge trial as they were in lay whereas the birds in the pilot study were 6 weeks old when challenged. The stresses of lay and/or the calendar age of the birds could have made the birds more susceptible to SG infection. Age does seem to play a role in the susceptibility of poultry to SG infection as natural disease is more common in layers (Wigley, Hulme, Powers, Beal, Smith and Barrow, 2005b). As a result, it

was decided that with the next two challenges at 16 and 24 weeks post-vaccination, the SG challenge dose would be reduced to 1 x 10^8 cfu's.

Figure 16 shows the MDT of birds dying from FT infection over the three challenge periods. In the control birds, as they were not vaccinated, we expected a constant MDT over the three challenges. In fact, the MDT increased in the second challenge when compared to the first. This is explained by the reduction in the challenge dose between the birds in the first and second challenges. In the third challenge, the MDT decreased when compared with the birds in the second challenge, this decrease was not significantly different ($P>0.05$). The MDT of the injected birds decreased with the second challenge; however this decrease within the injected group was not significantly different from the first challenge ($P>0.05$). This minor decrease could be due to the low numbers of mortalities in this group that may have skewed the results, or that the vaccine protection had decreased slightly over the 8-week period between challenges. The MDT of the orally vaccinated birds increased during the second challenge, this was expected in both vaccinated groups due to the decreased challenge dose, and this increase however, was not significantly different from the first challenge ($P>0.05$). In the third challenge the MDT in both vaccinated groups decreased substantially, both groups were significantly different from their previous challenges ($P<0.05$). This was due to the diminishing vaccine protection.

The OBPft vaccine protected birds well against challenge with virulent SG for at least 16 weeks after vaccination. By 24 weeks post-vaccination the level of protection had declined to 30 %. Other live SG vaccines claim good protection up

to 12 weeks post vaccination (Young *et al*., 2005). Thus the OBPft vaccine shows good protection for 4 weeks longer whether given orally or via injection. In fact, the duration of protection may even be a few weeks longer as the next challenge was only done 8 weeks later.

The injected and oral groups both showed a 70 % protection from mortality in the challenge done 16-weeks post vaccination using 1 x 10^8 cfu's of the SG challenge strain. These results were similar to those seen in the previous challenge at the higher dose.

When Cameron and Buys (1979) did similar work on 8 – 10 week old New Hampshire chickens with the vaccine applied by injection, they obtained protection of between 80 and 100 % up to 8 weeks post vaccination. This was done using 2.5 x 10^8 cfu of SG challenge strain 1007.

When Cameron and Buys (1979) then challenged birds 16 weeks post-vaccination they found only 50 % protection. This shows that the protection we observed in our 16 week post vaccination challenge was in fact superior, and allows for extrapolation; if our birds were challenged with the lower dose at 8 weeks post-vaccination our percentage protection may have been in the 80 – 100 % range. In this trial, however, the comparison between the two routes of vaccine administration is more important than the protection offered and our results show they are comparable.

In the field, challenges of the magnitude used in this project are highly unlikely in vaccinated flocks, due to the lack of shedding observed by the vaccinated groups in this project. With a decreased build up of SG in vaccinated flocks a better vaccine protection may be observed.

5.2.4 Necropsy

The spleen, liver and lungs showed the most marked pathological changes in this trial. The spleens were enlarged, some with white pulp hyperplasia, the livers were also enlarged with yellow/brown discolouration, and the lungs were pale to grey/white in colour. SG was recovered from these organs as expected, in birds that had died.

Egg peritonitis caused by *E. coli* was common in the surviving vaccinated birds with 15.5 % being affected. This ubiquitous bacterium is a common cause of opportunistic infections in birds suffering from other diseases (Saif, 2003). It was the eggs from the surviving unvaccinated control birds if any that we were hoping to test for the potential vertical transmission of SG, however there were no survivors. All the eggs cultured from vaccinated groups were negative for SG, indicating that vertical transmission of SG did not occur in this experiment. These findings correlate with Berchieri, de Oliveira, Pinheiro and Barrow, (2000); where they too could not prove vertical transmission of this disease in challenged but unvaccinated birds.

5.2.5 Egg production

The percentage eggs produced was calculated using the hen day egg production method, this method counts the eggs produced per day by the number of birds available to produce eggs. Thus, dead birds are excluded from the equation (Table 21 and Figure 13).

A marked drop in egg production was noted directly after the challenge; the reason for this drop is not clear and I suggest it may have been due to the handling of the birds. The egg production did increase over the next few days, however, not to the pre-challenge count except in the orally vaccinated group. The literature suggests that an egg production drop of 20 % should be expected in the event of a Fowl Typhoid outbreak (Saif, 2003) in a vaccinated flock. The production drop in the vaccinated groups was between 30 – 40 %, and the lowest values were around seven days post challenge. The production did increase again, and by 14 days post challenge the orally vaccinated group was producing eggs as before challenge.

The production drop in the unvaccinated control group was severe. By four days post-challenge the production figures had dropped to 20 %. Then the birds started dying and by 6 days post-challenge no eggs were being produced by the unvaccinated control group. This group never recovered, by nine days post-challenge all the birds in this group were dead (Table 21).

The egg production figures from the third challenge were not incorporated in this data, due to the lack of protection offered by the vaccine. Those results if added would skew the data and make the extrapolations less accurate, as we were testing the egg production drops in birds protected by vaccination, not where the vaccine was no longer protective. These production drops were higher than expected, however, this indicates that the challenge was much stronger, and the challenge dose much higher than one would expect from a natural infection. In a field challenge not all the birds in a flock would be challenged concurrently as in an experimental challenge, hence the egg production drop would probably be more gradual than seen here (Table 21).

The statistics showed that there was no significant difference in egg production between the two vaccinated groups at a 95% CI. However there was a significant difference between the vaccinated groups and the control group ($P<0.01$). With the statistical testing, in order to ensure that the significant difference seen was viable, an additional test was done excluding day -1 and day 0, as the challenge had not affected the birds yet and the last four days were removed to limit the potential skewing of the data due to extra days where the control group had no birds available to lay. A minimum of 10 samples were needed for the test, hence day 1 to 10 was used (Table 21). The results were the same as using all the data, as both vaccinated groups were still significantly different from the control group ($P<0.01$).

5.2.6 Duration of protection (conclusion)

The lack of evidence in the shedding of SG in eggs or faeces whether vaccine or challenge strain during this trial suggests that vertical spread of SG in birds vaccinated orally or via injection with the OBPft vaccine is highly unlikely. This could be due to our culture technique, maybe the addition of PCR testing and more frequent testing early on in the challenge might give different results.

Vaccination via the oral route with the Onderstepoort Biological Products Fowl Typhoid vaccine gives as good protection against experimental SG challenge as the injected route of vaccination does.

The Onderstepoort Biological Products Fowl Typhoid vaccine gives good protection up to 16 weeks post-vaccination. The available data indicates that the birds be vaccinated at 10 and 14 weeks of age, as well as given a booster vaccination at 30 weeks old to ensure further protection.

5.2.7 Future work

A field trial may be done to confirm these experimental results.

Perform a set of challenges a week apart over the 8 week period from 16 to 24 weeks post-vaccination to more accurately determine how quickly the protection decreases.

The trial could possibly be repeated using a lower challenge dose as the high dose has resulted in unacceptably high mortalities and drop in production. It would also allow one to investigate vertical transmission of SG via the eggs. More clinical parameters may be adopted, for example, the use of subcutaneous temperature probes and body weights.

The work done on the haematocrit tests in the pilot study did not show any variations, nor did it validate work done previously on anaemia being caused by SG infection (Assoku and Penhale, 1974). Our intention was to test whether it was possible to see early signs of infection by haematocrit tests, this could possibly be pursued in further studies.

Tests could be performed trying earlier vaccination to allow for less conflict in the vaccination schedule of pullets. Young *et al*. (2005); tested the SG9R vaccine, also a live SG vaccine and suggested that vaccination as early as 4 weeks was safe. This could be done, but duration of protection becomes a more important aspect here.

Flocks are, however, routinely kept to 70 weeks of age in the case of layers; hence, further testing would have to be done, to test the effect of the booster vaccination on birds in lay.

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