FOWL TYPHOID
THE DISEASE, IMMUNITY AND CONTROL

By

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DECLARATION

a) This thesis is my original work and has not been presented for a degree in any other University.

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b) This thesis has been submitted for examination with my approval as University Supervisor.

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LIST OF ABBREVIATIONS

A.O.A.C. - Association of Official Agricultural Chemists Analytical ?

BGA - Brilliant green agar

BSA - Bismuth sulphite agar

cc - Cubic centimetre(s)

CFT - Complement fixation test

DCA - Desoxycholate citrate agar

DTH - Delayed-type hypersensitivity

gm - gramme(s)

H-DPB - Heparinised calcium-magnesium-free Dulbecco phosphate buffer

HEA - Hektoen enteric agar

IHA - Indirect haemagglutination test

I.P. - Intraperitoneally

LD_{50} - 50% lethal dose

LI sLant - Lysine iron sLant

MAGT - Macro-or Micro-antiglobulin test

MAT - Microagglutination test

MCA - MacConkey agar

MEM - Minimal essential medium

mg - Milligramme(s)

mg/ml - Milligramme(s) per millilitre

MIF - Migration inhibition factor

MIT - Macrophage migration inhibition test

ml - Millilitre(s)

mm - millimetre(s)

org/ml - organisms per millilitre

org/g - organisms per gramme of tissue

PBS - Phosphate buffered saline

PT - Precipitin test

rbc - red blood cells

RSPT - Rapid serum plate test

RTD - Routine test dilution

RWBPT - Rapid whole blood plate test

LIST OF ABBREVIATIONS

SAT	-	Slide agglutination test
SB	-	Selenite broth
SSA	-	Salmonella - shigella agar
TAT	-	Tube agglutination test
TB	-	Tetrathionate broth
TSI	-	Triple sugar iron agar
µl	-	microlitre(s)
wt	-	weight
WT	-	wattle test
XLDA	-	Xylose lysine deoxycholate agar
% MI	-	Percent migration inhibition

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SUMMARY

Although vaccination against fowl typhoid is done widely in Kenya, some of the mechanisms pertaining to pathogenicity of *Salmonella gallinarum* isolates, their pathogenesis and the host's immune response to infection are not clearly understood. At times, the disease has been observed in flocks which had been vaccinated with the live vaccine. This necessitated the study of various aspects of the fowl typhoid disease with a view of discovering the cause of the vaccination breakdowns. These aspects included :- the study of the disease in general, the study of the immune response to *S. gallinarum* isolates and the study of the various ways of controlling the disease. A new vaccine was developed and the bird's immune response to it studied. This was compared with the immune response induced by the currently used vaccine strain, CN 180.

Isolates from fowl typhoid outbreaks were characterised biochemically and serologically. Subsequently, their sensitivity to antibacterial agents was tested. This included antibiotics, disinfectants and phages. Virulence testing (LD_{50} - calculations) was also done. All this was carried out in search for markers for *S. gallinarum* bacteria and also to evaluate the possibility of occurrence of different strains. The pathogenicity of *S. gallinarum* isolates was studied in birds at various age groups

Twenty (20) isolates at various doses ranging from 10 to 10^9 organisms per millilitre were used to challenge different groups of day-old-chicks intraperitoneally. Adult birds were challenged with a pool of 3 *S. gallinarum* isolates at concentrations ranging from 50 to 10^6 organisms per millilitre through intraperitoneal and oral routes. The pathogenesis of *S. gallinarum* in the host was studied by dosing 2 groups of 45-day-old unvaccinated birds intraperitoneally and orally, separately, with *S. gallinarum* isolate L41 at the dosage of 9.6×10^7 organisms per millilitre and 3.2×10^9 organisms per millilitre, respectively. The birds from each group were then killed, two at a time, at various intervals and their heartblood, livers, spleens, caeca processed for bacterial isolations.

To elaborate on the diagnosis of fowl typhoid disease, a study of the relationship between the immune status of an infected bird and any one of the three *S. gallinarum* somatic antigens was carried out. This was done by collecting sera from various birds that were suffering or had suffered from fowl typhoid outbreaks and monitoring the antibody titres using indirect haemagglutination test. The sera were screened with the various antigens, separately. To confirm the field findings, cockerels were experimentally vaccinated with CN 180 and their humoral and cellular immune responses were monitored by indirect haemagglutination test and macrophage migration inhibition test, respectively. The specimens were screened with the various antigens separately.

From the LD_{50} experiments, isolate L46, with an LD_{50} of 1 organism was chosen as the experimental vaccine strain. Preliminary studies showed that at a dose of 1,000 organisms per adult bird intramuscularly, L46 behaved as if it was non-pathogenic to the birds. This dosage was used henceforth to study the immune response to L46 as a model for *S. gallinarum* infections. A concurrent study of the currently-used vaccine strain, CN 180, was also carried out. The humoral and cell-mediated immune responses were assayed using indirect haemagglutination test (IHA) and macrophage migration inhibition test (MIT), respectively. Birds were vaccinated with the 2 vaccines, respectively, and bleeding for both sera (for IHA) and cells (for MIT) was done every week up to 7th week post-vaccination, then, after every alternate week 3 times, and later once every month for a total period of 37 weeks.

For the control of the disease, the ability of the vaccines to protect pullets from the disease was studied. This was monitored by challenging the vaccinated birds with a virulent strain of *S. gallinarum* and calculating the liver and spleen hypertrophy indices and the liver clearance capacity of the respective birds. The calculations were made after sacrificing a few birds after one, two, three and six days post-challenge and recording their body weights, liver weights, and spleen weights. The antibiotic and disinfectant sensitivity tests were used to survey the various antibacterial agents that could be utilized to control fowl typhoid.

The findings indicated that the isolates recovered and characterized by the various methods were different strains. The pathogenicity study done with day-old chicks indicated that various isolates had different pathogenicities. The adult birds infected orally did not show any signs of disease while those infected intraperitoneally died at a rate proportional to the dosage given. The post-mortem lesions of the dead birds were classically the same as those reported in literature on fowl typhoid disease. The currently-used vaccine strain, CN 180, was confirmed to be non-pathogenic to day-old chicks. The study on the pathogenesis of *S. gallinarum* in the host bird showed that the intraperitoneally-inoculated birds had organisms in their livers and spleens as early as 3 hours post-inoculation and these could be detected up to 7 days post-inoculation. The organisms were not detected in the caeca of these birds. The orally inoculated birds had organisms in their crops up to 6 hours post-inoculation but none was detected in the gizzard, duodenum and caecum throughout the experimental period. These birds had organisms in their livers and spleens 24 hours post-inoculation after which the numbers fell to below detectable levels.

In studying the relationship between the immune status of an infected bird (IHA titre or MIT) and any one of the three *S. gallinarum* somatic antigens, it was observed that the 3 antigens gave rise to varying degrees of immunity as shown by IHA titres and degree of macrophage inhibition

(MI), but there was no constant pattern relating higher IHA titres or higher degrees of MI to one particular antigen.

Strain L46 was found to induce an immune response that was very similar to that of CN 180, although there were times when it showed superiority. Both gave good humoral and cellular responses.

The liver and spleen hypertrophy indices and liver clearance rates compared well between L46 and CN 180 - vaccinated birds, which showed that both produced a good immunity which got rid of the *S. gallinarum* organisms as soon as they entered the body. The antibiotic sensitivity testing showed that antibiotics that had good effect in controlling the disease were furazolidone, neomycin, chloramphenicol, tetracycline, ampicillin gentamycin and nitrofurantoin. The effective disinfectants for controlling *S. gallinarum* contaminations were Bromosept, lysol and biodan.

This study indicates that the various *S. gallinarum* isolates studied are of different strains. The difference is either in their biochemical processes as indicated by their reaction to the antibiotics and disinfectants used; or in their phage-receptors, as indicated in the phage typing experiment where they showed differing inhibition-zone diameters. The varying degrees of immunity to the various *S. gallinarum* somatic antigens as shown in the study on the relationship between the immune status of an infected bird and any one of the three *S. gallinarum* somatic antigens could either be due to a difference in the amounts of antigens exposed by the isolates surveyed, or due to a difference in individual chicken's immune response towards the various somatic antigens. This, together with the possible emergence of new virulent strains of *S. gallinarum*, may explain the occassional breakdown of vaccinations with the live-attenuated vaccine, CN 180. The virulence and pathogenicity tests indicated varying virulence as shown by the mortalities in the various groups tested. This indicates a strain difference although one should note, here, that any reduced virulence may be due to differences in the maintenance of different isolates on artificial media, resulting in varying degrees of attenuation.

For self defence, the host initiates both humoral and cellular immunity which gets rid of the organisms. The new vaccine produced, L46, compared well with the currently-used vaccine strain, CN 180, in their induction of immune response and protection to disease; and in some cases, it has shown greater efficiency.

To control the disease, treatment using the various antibiotics given above is possible, but it should be noted that most of the survivors tend to remain carriers of the disease organisms. Disinfection is also possible. For prophylaxis, vaccination has proved more convenient and this study has shown that both L46 and CN 180 can be used as the vaccine strains. They give good immune responses.

INTRODUCTION

Fowl typhoid, caused by *Salmonella gallinarum*, is one of the most serious diseases of poultry. The disease causes high mortality in chickens of all ages and reduced egg-production, fertility and hatchability. *S. gallinarum* infection in surviving chicks can cause stunted growth to the extent that infected birds are of low quality when they reach market age. The survivors normally become carriers (Pomeroy, 1972).

S. gallinarum organisms are gram-negative rods, closely related morphologically and physiologically to other genera of the family *Enterobacteriaceae*. They are non-motile and produce acid only (no gas) from glucose, maltose, dulcitol and mannitol, but not from sucrose and lactose (Bergey's manual of Determinative Bacteriology, 1974). Majority of the isolates have been easy to treat. The treatments of choice are furazolidone, neomycin, furaxol, neolidone and chloramphenicol (poultry clinic, University of Nairobi, unpublished data). However, these isolates have shown varying patterns of resistance to various antibiotics, more so to tetracyclines, streptomycin and sulphonamides, necessitating carrying out of antibiotic sensitivity-testing for each isolate per outbreak before treating.

When infected, a bird initiates both humoral and cellular immunity against the causative agents (Pomeroy, 1972). While cell-mediated-immunity has been shown to be the one responsible for protection against invading *Salmonella* organisms (Collins, 1972; 1974; Dannenberg, 1968; Mackaness and Blanden, 1967), humoral immunity is also known to play a role in resistance against *Salmonella* infections (Jenkins and Rowley, 1965; Rowley *et.al*, 1968; Cameron, 1976; Davies and Kotlarski, 1976; Hochadel and Keller, 1977).

A survey done on avian salmonellosis in Kenya (Bebora *et.al*, 1979) indicated a high incidence of the disease. The same is indicated in the microbiology diagnostic laboratory reports at Veterinary Research Laboratories, Kabete and at the Faculty of Veterinary Medicine, Kabete. Since poultry-keeping has become a significant agro-industry, and poultry-products are an important source of protein in Kenya, all possible measures should be taken to protect poultry populations from avian salmonellosis.

Vaccination programmes have been carried out in Kenya using both live and dead fowl typhoid vaccines administered intramuscularly at 8 weeks of age. The live vaccine gives better protection than the dead one (Winmill, 1961), although there have been cases where vaccinated birds succumbed to fowl typhoid infections. There was, therefore, need to study various aspects of the fowl typhoid disease with a view to discovering the cause of the vaccination breakdowns.

This study covered three aspects of the disease:- the pathogenicity and pathogenesis of selected isolates, the immune response to the *S. gallinarum* isolates, and the various ways of controlling the disease. A new vaccine strain was developed and the birds' immune response to it studied. This was compared with the immune response induced by the currently-used-attenuated vaccine strain, CN 180.

2. REVIEW OF LITERATURE

2.1 THE S. GALLINARUM AND OTHER SALMONELLA BACTERIA

2.1.1 History

The etiological agent of fowl typhoid, *Salmonella gallinarum*, was first isolated in 1888 in England and was named *Bacillus gallinarum* by Klein (1889). Moore (1895) investigated the disease in Virginia and Maryland (USA) and named it "infectious leukemia" and the causative agent *Bacillus sanguinarium*. It was Curtice (1902), studying the disease in Rhode Island (USA), who named it "Fowl Typhoid". Currently, the disease is known to occur throughout the World (Williams, 1972, 1978).

Fowl typhoid has been reported to cause heavy losses in Kenya and Tanganyika (Lowe, 1932) and in Uganda (Hall, 1926). This disease was first reported in Kenya by Montgomerie (1911 to 1912). Since it was first encountered in the Kikuyu district, it was referred to as "Kikuyu Fowl Disease" (KFD). The disease continues to cause heavy losses to chickens to-date (Report, 1976-1985 a, b).

A very similar disease, pullorum disease, caused by *S. pullorum*, was discovered by Rettger in 1899 in the United States. He described the disease as "Fatal

Septicaemia of young chicks" in 1900. The disease was also called "White diarrhoea" and "Bacillary white diarrhoea" successively. However, in 1932, the term "Pullorum disease" came into common usage (Van Roekel, 1952). Pullorum disease has been recorded in Kenya (Annual Report, Dept. Agric. Kenya, 1933), in Tanganyika (Lowe, 1932) and in Uganda (Mettam, 1932). It is interesting to note that the pullorum disease agent has not been isolated in Kenya since 1958 (Miringa, 1984) and it is, therefore, taken as being non-existent in Kenya. *S. pullorum* is taken as a strain of *S. gallinarum* (Bergeys Manual of Determinative Bacteriology, 1974).

Salmonellae other than *S. pullorum* and *S. gallinarum* cause paratyphoid infections. These infections may have existed for many years but the early workers did not have the present-day methods available by which the isolated organisms could be positively identified. Mazza (1899) described a chicken epizootic that raged in various parts of Northern Italy. He isolated the causative organism which may have been a paratyphoid. Other workers, for example, Henning (1939) Buxton (1957), Khan (1970) and Shigidi (1973) described the disease in chickens and showed that paratyphoid infections were common among chickens and had a Worldwide distribution.

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2.1.2 The Biology of Salmonella gallinarum

Salmonellae are gram-negative rods, closely related morphologically and physiologically to other genera of the family *Enterobacteriaceae*. They are usually motile although non-motile forms occur. *S. gallinarum* and *S. pullorum* are not motile. Most strains produce acid and gas from glucose, maltose, mannitol and sorbitol except *S. typhi* and *S. gallinarum* which produce acid only. Anaerogenic strains of normally gas-producing serotypes are found in nature. This is particularly so with *S. dublin*. The anaerogenic nature differentiates *S. gallinarum* from the closely related *S. pullorum*, which produces acid and gas from sugars. Salmonellae do not ferment sucrose, salicin and lactose. However, lactose-fermenting species have been isolated (Easterling *et.al*, 1969, Kristensen, 1955). Salmonellae do not form indole, do not coagulate milk or liquify gelatin. Their optimum temperature is 35 to 37°C, although some possess unusually high heat-resistant properties (Erskine and Margo, 1974). *S. gallinarum* is easily killed by exposure to 60°C for 10 minutes.

In the environment, salmonellae can remain viable for a long time. Survival of 87 days in tap water, 115 days in pond water, 120 days in pasture soil, 280 days in garden soil, over 30 months in dried bovine manure, 28 months in naturally infected avian faeces and 47 days in manure slurry has been reported (Erskine and Margo, 1974). *S. gallinarum* remains viable in the dark and at room temperature for 20 days in ordinary and distilled water, but dies in 24 hours when exposed to sunlight. When dried on glass plates and kept in the dark, the organism retains its viability for 89 hours. It has also been ascertained that it can live for years in the soil (Pomeroy, 1972). Orr and Moore (1953) tested *S. gallinarum* for longevity under various conditions and found that in cloth, in the dark and at room temperature, the organism remained alive for 228 days. On plastic cover-slips, some *S. gallinarum* organisms were viable up to 93 days. They retained viability up to 43 days when subjected to daily freezing and thawing. Tucker (1967) found that *S. gallinarum* persisted in built-up litter from 3 weeks in old litter to 11 weeks in new litter. When the infected pens were left unoccupied, the survival time in both types of litter was increased to more than 30 weeks.

The antigens of *Salmonella* species are divided into two broad groups:- (i) 'O' - antigens and (ii) 'H' - antigens. 'O' - antigens are bacterial cell-body antigens. They are further divided into:- (a) 'O' antigens proper or somatic antigens, which are composed of bacterial polysaccharide and are prepared by heating the bacterial suspension for 2½ hours at 100°C or by extraction with hot alcohol, and (b) 'K' antigens, which are either envelope or capsular antigens. K - antigens occur in many groups of *Enterobacteriaceae*. The term "K-antigen" denotes a group of different capsular and envelope antigens which include A, B, L, Vi antigens, 5-antigen, M-antigen, fimbriae antigen etc. which have different biochemical properties. The 'K'-antigens can confer inagglutinability of the 'O' antigens proper. Proper heating is required to inactivate these and allow normal 'O' agglutination to occur. 'O' antigens are designated by numerals i.e. 1,2,3,4 etc. Testing with factor sera (i.e. sera containing antibodies against one particular antigen) one can screen the bacteria and find what antigens it contains eg. 3, 9, 10 - this will form an 'O'-antigenic formula for the particular organism (Kauffmann, 1975).

'H'-antigens are flagellar antigens. These are prepared by subjecting the bacterial suspension to formalin, which fixes the flagella over the surface of the bacterium, thereby covering the 'O' antigens of the cell-body. The 'H' antigens are heat-labile proteins. Flagellar antigens are not as many as the 'O' antigens, and they occur in two phases designated as "specific" and "non-specific". The specific phase is composed of only those antigenic components that are specific for the species or strain of the organism. These antigens are designated a, b, c etc. The non-specific phase is represented by the antigens shared by other species, in other group types. The antigens are designated 1, 2, 3, 4 etc. So, by usage of factor sera, an 'H' antigenic formula can be ascertained. It will include both specific and non-specific 'H'-antigens eg. a, b: 1, 2 (Kauffmann, 1975).

Combining the two formulae, one gets a complete formula, including both 'O' and 'H' antigens, and this classifies the organism. *S. gallinarum* possesses the 'O' antigens 1, 9, 12 (Kauffmann, 1975). It has no 'H' antigens since it is not flaggellated.

Smith and Ten Broeck (1915) found a toxin in broth culture filtrates of *S. gallinarum*. It appeared in the culture at the end of 2 days after incubation at 37°C and caused prompt death to a rabbit when injected by the intravenous route. Death resulted within 2 hours and, in many aspects, was like an anaphylactic shock. It was probably an endotoxin which was stable at 60°C for one hour. Boiling for 15 minutes reduced its activity.

2.1.3 Isolation and Identification of the Organism

In the intestinal tracts of man and animals, there are a number of different microorganisms. This presents a complex problem in isolating salmonellae. Thus, a culture medium, inoculated with a loopful of intestinal contents, normally yields a very mixed culture. For this reason, to ensure that *Salmonella* bacteria, if present, can be isolated, special media have been devised for their isolation. In a severe, enteric *Salmonella* infection, it is not necessary that intestinal contents from such an animal be inoculated into a preliminary enrichment medium, since it can be assumed that the population of salmonellae in such a specimen is high and that they should be fairly easy to isolate. However, there are times, such as when surveys of the occurrence of *Salmonella* are carried out, that the number of bacteria in the intestinal tract may be so low as to make it a prerequisite to first inoculate the samples into enrichment media. Media used for this purpose are selenite broth (SB) and Tetrathionate broth (TB) (Merchant and Packer, 1967).

Andrews *et.al.* (1977) compared methods for the isolation of *Salmonella* species from frogs' legs. They did not find a significant difference between numbers of salmonellae isolated through SB and TB. Harvey *et.al* (1977) carried out a similar study, but in pigs, and confirmed the findings of Andrews *et.al.* (1977). Jameson (1961) found that a secondary selective enrichment medium inoculated with a relatively large inoculum from the primary enrichment medium, increased the yield of salmonellae.

The commonly-used selective media for salmonellae are:- Salmonella-Shigella agar (SSA), Bismuth Sulfite Agar (BSA), Desoxycholate Citrate Agar (DCA) and MacConkey Agar (MCA) (Merchant and Packer, 1967). The selective media are not equally effective in the isolation of salmonellae. Andrews *et.al.* (1977) compared the effectiveness of SSA, Brilliant Green Agar (BGA), BSA, Xylose Lysine Deoxycholate Agar (XLDA) and Hektoen Enteric Agar (HEA). They found that when SSA was streaked with material from either SB or TB it gave significantly fewer *Salmonella* than the other four media. The use of XLDA and HEA as a supplement of SSA, BGA and BSA enhanced the recovery of salmonellae.

On solid media, salmonellae form small, smooth, glistening colonies with entire or slightly undulated edges. On MCA and SSA, the colonies are normally pale in colour, since salmonellae are normally non-lactose fermenters. Owing to the fact that SSA contains ferric ammonium citrate, the colonies may have a black centre due to formation of hydrogen sulfide (H_2S). DCA also contains ferric ammonium citrate, thus, on this medium, *Salmonella* colonies are colourless, surrounded by a clear orange-yellow zone of medium and have black centres due to H_2S production. On BSA, salmonellae form typical discrete colonies which are black and surrounded by a black zone (due to H_2S production) which may be several times the size of the colony. By reflected light, this zone exhibits a characteristic metallic sheen.

Suspicious colonies are biochemically and serologically investigated. A list of all the possible biochemical tests that can be carried out is given by Cowan and Steel (1974), Carter (1975) and Merchant and Parker (1967). Cox and Williams (1976) have come up with a simplified biochemical system to screen *Salmonella* isolates. This includes inoculation of triple sugar iron (TSI) slant, lysine iron (LI) slant, and six fermentation broths which were numbered;- 1 (dextrose), 2 (lactose), 3 (sucrose), 4 (mannitol), 5 (maltose) and 6 (dulcitol). In their study, all the *Salmonella* cultures (except *S. Pullorum)* gave a 1, 4, 5, 6 code which means they produced acid and, in most cases, gas, in dextrose, mannitol, maltose and dulcitol, but no acid or gas in lactose and sucrose. *S. Pullorum* gave a 1, 4 code. All non-*Salmonella* cultures gave fermentation patterns different from the 1, 4, 5, 6 pattern of the paratyphoids and *S. gallinarum*.

Serotyping or serological classification includes screening the isolate with polyvalent 'O' *Salmonella* antiserum (i.e. including antibodies against all representative antigens of the organisms) and then with the various factor (antigen-specific) sera, to give an antigenic formula (Kauffmann, 1975).

It is interesting to note that incubating the cultures, especially the initial enrichment cultures, at 43°C rather than at 37°C appears to be more effective for the isolation of *Salmonella* from heavily contaminated samples like sewage and faeces (Harvey and Thomson, 1953). There is less contamination of the plating medium with other organisms when the higher temperature is used and the reason for this appears to be that the contaminating organisms are being inhibited rather than the *Salmonella* being specifically favoured (Carlson and Snoeyenbos, 1972; Smyser *et.al.* 1970; Carlson *et.al.* 1967; and Spino, 1966).

2.1.4 Preservation of Cultures

Salmonellae, like any other bacteria, can be maintained or preserved in a number of ways. There are long-term and short-term preservation methods. The short-term methods include:- (i) maintenance in reduced metabolic state, (ii) storage under liquid paraffin, (iii) storage in distilled water, (iv) drying in soil or sand, and (v) drying on silica gel or molecular sieves (Kocur, 1981; Hill, 1981). The long-term methods include:- (i) storage on Dorsett's egg medium, and (ii) storage by freeze-drying (Kocur, 1981, Hill, 1981).

2.1.5 Resistance to Antibiotics

At an international meeting organized by WHO in Geneva in 1977 (WHO Report, 1978) on the surveillance for the prevention and control of health hazards due to antibiotic resistant enterobacteria, the experts expressed their concern on the Worldwide increase in antibiotic resistance associated with the growing and frequent indiscriminate use of antibiotics in both man and animals. In recent years, resistant bacteria have given rise to several serious outbreaks of infection with many deaths (WHO report, 1979). A similar observation is made when one surveys the annual reports of the Bacteriology department, faculty of Veterinary Medicine, University of Nairobi (Report, 1976-1985a), where most of the salmonellae isolated show resistance to various antibiotics, more so to tetracyclines, streptomycin, penicillin and sulphonamides. This drug resistance is still being reported to date (Hinton, 1982; Datta, 1984; Chopra, 1984).

Bacterial resistance to antibiotics is the principal obstacle to their successful therapeutic use. When resistance develops during a course of treatment, it may deprive an antibiotic of its proper therapeutic effect in the "patient" being treated. More important, in the long run, is the effect on the general community, since the elimination of sensitive strains and the dissemination of resistant ones leads to a situation in which many infections cannot be treated and alternative treatment must be adopted. For this reason, the estimation of bacterial sensitivity or resistance to antibiotics has assumed great importance so as to lead the clinicians in selecting the best antimicrobial agent for an individual "patient" (WHO Rep., 1961).

To describe a microorganism simply as "sensitive" or "resistant" to an antibiotic, although a common practice, is inexact. Resistance is never absolute and calls for a quantitative expression. It is, therefore, preferable to say that a microorganism is sensitive (or resistant) to a specified concentration of the antibiotic. The WHO report (1979), suggests a grouping of antibiotic sensitivity into 3 groups:-

(i) Susceptible group: A microbe is called "susceptible" to a drug when the infection caused by it is likely to respond to the treatment with this drug, at the dosage recommended.

(ii) Intermediate group: The susceptibility of an organism is called "intermediate" when the infection is likely to respond to unusually high doses of the drug.

(iii) Resistant group: This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and of the location of the infection,

> OR it is able to withstand an appreciably higher concentration of the antibiotic than the concentration obtainable *in vivo*.

Genotypically resistant individual bacteria may arise by spontaneous mutation quite independent of the antibiotic. It has been confirmed by genetic analysis that a high degree of resistance may be the result of either a single mutation or a series of additive mutations (WHO report, 1961). These resistant mutants will then lead to the emergence of resistant clones, especially in chronic infections and during the indiscriminate prophylactic use of certain antibiotics. These resistant clones then progressively replace the sensitive microorganisms. Resistance can be transferred from one species to another through conjugation (Watanabe, 1963; Anderson, 1968).

Antimicrobial susceptibility tests measure the ability of an antibiotic or other antimicrobial agent to inhibit the *in vitro* bacterial growth. The ability may be estimated by either the dilution or diffusion method (WHO, Report, 1979). The dilution test is used for quantitative estimates of antibiotic activity. Dilutions of the antibiotic are incorporated into broth or agar medium which is then inoculated with the test organism. The lowest concentration that prevents growth after overnight incubation is known as the minimal inhibitory concentration (MIC) of the agent.

This MIC value is then compared with the known concentration of the drug obtainable in the serum and in other body fluids to assess the likely clinical response. This method is, however, too laborious and time-consuming for general clinical use. In the diffusion test, paper discs impregnated with the antibiotic are placed on agar medium uniformly seeded with the test organism. A concentration gradient of the antibiotic forms by diffusion from the disc, and the growth of the test organism is inhibited at a distance from the disc that is related, among other factors, to the susceptibility of the organism i.e. the inhibition zone diameter is directly related to the degree of organism's susceptibility to the antibiotic. The other adaptations of this test include usage of tablets saturated with the antibiotic or digging a well or hole into the agar and filling it with the antibiotic solution (WHO Rep. 1961).

A number of technical factors influence the size of the zone in the disc diffusion method. These are as given below (WHO Rep. 1979):-

(i) Inoculum density

If the inoculum is too light, the inhibition zones will be larger although the sensitivity of the organism is unchanged. Relatively resistant strains may then be reported as susceptible. Conversely, when the inoculum is too heavy, the zone size is reduced and susceptible strains may be reported as resistant. Usually, optimal results are obtained with sizes of the inoculum producing confluent or near confluent growth.

(ii) Timing of disc application

If the plates, after being seeded with the test strain, are left at room temperature for periods longer than the standard time, multiplication of the inoculum may take place before the discs are applied. This causes a reduction in the zone diameter and may result in susceptible strains being reported as resistant.

(iii) Temperature of incubation

Optimal temperatures are 35 - 37°C. If the temperature is lowered, the time required for effective growth is extended and larger zones result.

(iv) Incubation time

Most techniques adopt an incubation time of 16-18 hours. If shorter, smaller zones may result.

(v) Size of plates, depth of agar medium, and spacing of the antibiotic discs

Susceptibility tests are usually carried out with 9-10 cm plates and not more than six to seven antibiotic discs for each plate. If larger numbers of antibiotic have to be tested, two plates or plate with a diameter of 14 cm. are to be preferred.

This is because proper spacing of discs is essential to avoid overlap of inhibition zones or deformation near the edge of plates. Excessively large inhibition zones may be formed on very thin media. The converse is true for thick plates.

(vi) Potency of antibiotic discs

The diameter of the inhibition zone is related to the amount of drug in the disc. If the potency of the drug falls owing to deterioration during storage, the inhibition zones show a gradual reduction in size.

(vii) Composition of the medium

The medium influences the zone size by its effect on the rate of growth of the organism, the rate of diffusion of the antibiotic, and the activity of the agent. It is essential to use the medium appropriate to a particular method.

The many factors influencing the zone diameters that may be obtained for the same test organism clearly demonstrate the need for standardisation of the method before starting to work with it. The zone interpretative chart is given by McGhie and Finch (1975). Up to 2mm inhibition zones (measured from the end of the disc to the inhibition zone front) are taken as resistant. Zones that are beyond 2mm are taken as sensitive.

2.1.6 Methods of Testing the Efficacy of Disinfectants

A great variety of techniques have been employed in the laboratory by Microbiologists in studying the effects of physical agents and chemicals on fungi, bacteria and viruses since their role in the initiation of infection and disease was established in the latter part of 19th century by Semmelweiss, Pasteur, Koch and Lister (Stuart, 1968). Of the many *in vitro* laboratory methods presently employed in comparing germicidal chemicals one with another and providing an index to the concentration of products which can be employed in disinfecting inanimate surfaces where infectious organisms are suspected of being present, the Association of Official Agricultural Chemists' (AOAC) phenol coefficient method (1965) has been appraised more for its precision and accuracy (Stuart, 1968, Spooner and Sykes, 1972). However, it has been shown that, considering precision or reproducibility of results, variations ranging from ± 12% to ± 23% have been reported (Stuart *et.al.*, 1958; Klimeck and Umbreit, 1948). These results show that the precision of the procedure is not as good as many early investigators believed.

The phenol coefficient method is basically a dilution tube technique and it is affected by various factors including culture media, test culture maintenance routines, test culture exposure manipulations, subculture routines, temperature for test organism exposure and subculture incubations (Stuart *et.al.*, 1955). This method is designed to determine the highest dilution of a germicidal chemical which will kill the test organism within a series of time intervals under specified conditions. From the test results, and comparable results obtained at the same time with the pure chemical, phenol, a specific calculation is made to yield a product known as "the phenol coefficient number". This number is employed to calculate the dilution which might be presumed to be equivalent in germicidal activity to a 5% solution of the pure chemical (phenol) or the maximum dilution that can be relied upon to disinfect under conditions commonly encountered in actual use. The maximum dilution calculated is taken as that required for practical disinfecting applications (Bass and Stuart, 1968). The phenol coefficient method yields a result which can be interpreted only indirectly in terms of the concentration of the product necessary for actual disinfection (Bass and Stuart, 1968).

When the phenol coefficient test was first proposed, typhoid fever was the infectious disease causing the greatest number of human fatalities; so its causative agent, *Salmonella typhosa*, was taken as the test organism of choice for use in this method (Bass and Stuart, 1968). However, McCoy *et.al.* (1917), Shippen and Griffin (1923), Brewer and Ruehle (1931), Klarmann and Shternov (1936) and Ostrolenk and Brewer (1949) called attention to the limited value inherent in test procedures restricted to the use of a single test organism. The adoption of *Staphylococcus aureus* as a secondary test organism in the F.D.A. Method (1931) partially corrected for this deficiency, and it was employed with *S. typhosa* in the next official A.O.A.C. procedure (Bass and Stuart, 1968). Considering the vast number of pathogens present in environments, we now have many other dilution techniques, using various types of cultures, used to compare the efficacy of various disinfectants (Bass and Stuart, 1968; Spooner and Sykes, 1972).

Disinfectant activity can also be determined on solid media. This can be done either by pre-inoculating the medium with the test organism and allowing the disinfectant to diffuse into the agar and produce a zone of inhibition of growth; or by incorporating the disinfectant into the agar and assessing the ability of an organism to grow on the surface of such a medium (Spooner and Sykes, 1972). The disinfectant can be supplied in what is described as a "cup" or a ditch dug into the agar (Spooner and Sykes, 1972). In the agar cup method, a plate of sterile agar, poured to a depth of about 4 millimetres is allowed to set and a single cup, 15 millimetre diameter, cut from the centre of the plate with a sterile cork-borer. After allowing the agar surface to dry for an hour or so, in the incubator, a loopful of a suitably diluted broth culture of each of 6 to 8 test organisms is streaked radially from the cup to the edge of the plate, after which the cup is filled with the test preparation (disinfectant) and the plate incubated at 37°C. The extent of inhibition of growth from the edge of the cup gives a measure of the activity of the preparation, so that a comparison of its relative activities against the range of test organisms is readily obtained. By using several plates simultaneously, the activities of several preparations can be compared but only approximately (Spooner and Sykes, 1972). In the ditch plate test, the cup is replaced by a ditch cut in the agar along the diameter of the plate. The ditch is filled with the disinfectant to be tested and selected cultures are streaked across the agar surface at right angles to the ditch. The interpretation is then as with the cup test.

To assess the relative activities of several preparations, a variation of the agar cup method can be employed (Spooner and Sykes, 1972). In this, instead of streak-inoculating the surface of the agar, the inoculum is either introduced into the molten agar before it is poured, the organism thereby being distributed evenly throughout the depth of the agar; or it can be flooded on the surface of the agar (and excess culture liquid drained off), thus giving a uniform spread of organisms over the surface of the plate. In either case, only one test organism can be used to each plate. The test samples (disinfectants) are then filled into the appropriate number of 15 millimetre cups cut in the agar (upto 5 cups can be used in each plate). After incubation, a comparison of the zones of inhibition around each cup gives a measure of the relative activities of the samples tested.

Alternative surface diffusion tests also use either of the two methods of inoculation just described, but, instead of cut-out cups, the test sample is placed on the surface of the inoculated agar and the zones of inhibition around each sample compared. This can be done in various ways, namely:- beads, filter paper discs, small porcelain or steel cylinders ("penicylinders"). The beads are first touched on the test solution so that they pick up a fixed volume of the disinfectant and then placed on the surface of the agar. The filter-paper discs or pads, 6mm diameter, are either dipped in the test solution, drained for a fixed short period and placed on the agar; or a small measured volume of the disinfectant is put on the agar by a pipette and the disc placed on the volume of disinfectant. The small porcelain or steel cylinders are placed on the agar and a fixed volume of the test solution, 2 or 3 drops, put into each cylinder. If the preparation to be tested is solid or semi-solid, which does not soften and flow at 37°C, the sample can be placed directly on the surface of the agar.

A modification of this method (Sykes, 1965 - cited by Spooner and Sykes, 1972), which is more sensitive and gives more clear-cut results, uses a serum agar containing 0.5% of glucose and 0.5% of sterile calcium carbonate in even suspension. A single loopful of a chosen culture, covering an area of about 4 millimetre diameter, is placed on the agar surface and the area covered by the test preparation (disinfectant). If the organism used is able to ferment glucose, the result after incubation will be a clear plate (where the organisms have grown and caused the calcium carbonate to dissolve) with small areas of white underneath the test sample area (where the organisms have been inhibited and so failed to dissolve the carbonate).

2.1.7 Phage Typing

It has been found that in various members of *Salmonella* group, there exists a close relationship between the structure of the surface antigens and the sensitivity to bacteriophage. On the basis of such observations Craigie and Yen (1938) were able to devise a method for typing *S. typhi* by means of specific anti-Vi phages; and Craigie and Felix (1947) managed to divide *S. typhi* into 24 types and subtypes. Lilleengen (1947, 1948) showed that anti-'O' phages, which earlier were considered to be of non-specific character, could also be used in typing given species of *Salmonella* group. He managed to divide *S. typhimurium* into 24 types. Lilleengen (1952), working on *S. gallinarum* and *S. pullorum*, managed to divide each of them into 6 distinctly differentiated types. The phage-typing method has been found to be of great value also in epidemiological and epizootiological investigations of outbreaks of infections (Barker and Tyc, 1982).

Before doing the actual phage-typing, the phage has to be propagated to give high titres, and also to be standardised (Corbel and Thomas, 1980; Rovozzo and Burke, 1973). In the propagation of phages, it is essential to control a number of factors which may influence the final product. Foremost among these is the propagating strain. This should be selected for each phage by examining a number of host strains and choosing one which consistently gives a high yield. In the interests of safety, a strain of low virulence should be chosen whenever possible (Corbel and Thomas, 1980; Rovozzo and Burke, 1973).

To produce maximum phage yields, it is essential to employ the optimum phage: bacterium ratio. This has to be determined for each phage and for each propagating strain. The conditions of incubation should also be standardised since sub-optimal conditions may favour mutation and dissociation in the propagating strain. In particular, where liquid medium is used, aeration should be well-maintained, the PH controlled and accumulation of bacterial metabolites prevented (Corbel and Thomas, 1980; Rovozzo and Burke, 1973). For propagation on solid media, the accumulation of excessive surface moisture should be avoided and a well-buffered, nutritionally adequate medium used. The use of antibiotics or other inhibitory agents in phage-propagating media should be avoided (Corbel and Thomas, 1980; Rovozzo and Burke, 1973).

There are three basic methods recommended for phage propagation. These are: (i) growth in liquid cultures, (ii) growth in agar overlay cultures, and (iii) growth in agar surface cultures. Details on how these procedures are carried out, and harvesting of the phages are given by Corbel and Thomas (1980). Corbel and Thomas (1980) noted that growth in liquid cultures gave rather low phage-titres while growth in overlay cultures gave high phage-titres.

The phage harvest needs to be standardised to give the plaque-forming units per millilitre, as well as the routine test dilution (RTD) (Corbel and Thomas, 1980, Rovozzo and Burke, 1973). The RTD is defined as the highest dilution of the phage stock which will produce confluent lysis of a lawn inoculum of the propagating strain. The RTD is determined by serial ten-fold dilutions as given by Corbel and Thomas (1980) and by Rovozzo and Burke (1973). This can be done as a streak or as a pour-plate method.

Adsorption of phage is influenced by many factors including the composition of the medium, temperature, physiological condition of the host cells etc. (Corbel and Thomas,1980; Rovozzo and Burke, 1973). Optimal conditions will, therefore, vary with each phage. Sodium ions are often necessary in media, for phage adsorption, and the presence of tryptone leads to rapid increases in the number of some phage particles. Incubation temperatures vary with the bacterium used although 37°C is standard.

Phage-typing technique can be done either by streak or pour-plate methods as given by Corbel and Thomas (1980) and by Rovozzo and Burke (1973). The reading is based on lysis of the bacterial cells; so, in a "lawn" of bacterial cells, there will be clear areas or plaques, indicating areas of lysis or phage activity. These can easily be seen against the "lawn" of bacteria. If streak-method is used, the plaques are graded 1+ to 4+ depending on the clarity of the plaques, (Corbel and Thomas, 1980).

2.2 THE DISEASE CAUSED BY S. GALLINARUM

2.2.1 Introduction

The pathogenicity of fowl-typhoid cultures has proved decidedly variable in the hands of different investigators, probably because they used cultures varying widely in virulence. Like most pathogenic microorganisms, *S. gallinarum* loses virulence rapidly on artificial media (Pomeroy, 1972). Hence cultures of *S. gallinarum* should be passaged serially in their natural host, the chicken, before testing the pathogenicity of the organism. The pathogenicity of such cultures is best maintained in the lyophilised or frozen state. With a uniformly pathogenic culture, most commonly used routes of exposure to chickens prove fatal (Pomeroy, 1972).

Rao *et.al* (1952) reported *S. gallinarum* to be equally pathogenic to susceptible baby chicks and adult birds under normal conditions. The incubation period is 4-5 days, although this varies with the virulence of the organism. The course of the disease is about 5 days and, in a flock, the losses from the disease may extend over a 2-3 week period with a tendency for recurrence.

2.2.2 Clinical Signs and Post-Mortem Lesions

Although the disease is encountered more frequently in growing and adult chickens, it may be encountered in young chicks, resulting from egg-transmission (Pomeroy, 1972). The signs noted in young chicks as given by Pomeroy (1972), are as follows:- if birds are hatched from infected eggs, moribund and dead chicks may be observed in the incubator. Within a short time after hatching, they manifest somnolence, weakness and loss of appetite, and death may follow suddenly. In some instances, evidence of the disease is not observed until several days (5-10) after hatching. The disease gains momentum during the following 7-10 days. The peak of mortality usually occurs during the 2nd or 3rd week of life. In some instances, the birds exhibit lassitude, an inclination to huddle together under the hover, loss of appetite, drooping of wings, somnolence and a distorted body appearance. Affected birds frequently exhibit a shrill cry when voiding excreta and commonly develop an accumulation of chalky-white excreta, sometimes stained greenish-brown, in and around the vent. Laboured breathing or gasping may be observed as a result of extensive pathology of the lungs. There has been reported cases of blindness and arthritis associated with salmonellosis. Survivors may be greatly retarded in their growth and appear underdeveloped and poorly feathered. However, some survivors may not reveal any great setback in growth but develop to maturity even though harbouring the infection. Flocks which have passed through a serious outbreak usually reveal a high-percentage of carriers at maturity.

In adults, an acute outbreak of disease in chickens may begin by a sudden drop in feed consumption with birds showing droopy wings, ruffled feathers and having pale and shrunken combs. The birds' temperature may increase by 2-5°C within 2-3 days after exposure and remain high until a few hours before death. There is usually greenish-yellowish diarrhoea. Death may occur within 4 days after onset of the disease, but usually in 5-10 days. In less-acute cases, the laying birds show a drop in egg-production, and breeding birds show a drop in hatchability and fertility (Pomeroy, 1972).

In chicks, that die suddenly in the early stages of brooding, the lesions are limited. The liver may be enlarged and congested, and the normal brown colour may be streaked with haemorrhages. In the septicaemic form, petecheal haemorrhages may be found in all the organs. The yolk sac and its contents normally reveal slight or no alteration although, in more protracted cases, an interference with yolk absorption may occur, and the yolk sac contents may be brownish in colour and of creamy and cheesy consistency. Necrotic foci or nodules may be present in the cardiac muscle, liver, lungs, caeca, large intestine, and the muscles of the gizzard. Pericarditis may be observed in some cases. The liver may reveal punctiform haemorrhages. The spleen may be enlarged and the kidneys congested or anaemic with ureters prominently distended with urates. The caeca may contain a cheesy core, sometimes tinted with blood. The wall of the intestines may be thickened and its contents fluidy. Frequently peritonitis is manifested. Among chicks only a few days old, the lung lesions may consist only of a haemorrhagic pneumonia, whereas, in older chicks, yellowish gray nodules and areas of gray hepatisation may be found. The nodules in the myocardium may attain sufficient size to cause a marked distortion of the shape of the heart (Pomeroy, 1972).

In adults, the lesions found most frequently in chronic carrier hen are misshapen, discoloured, cystic ova, peritonitis and frequently acute or chronic pericarditis. The diseased ova, usually contain oily and cheesy material enclosed in a thickened capsule. These degenerated ovarian follicles may be closely attached to the ovary, but frequently they are pedunculated and may become detached from the ovarian mass. In such cases, they become embedded in the adipose tissue of the abdominal cavity. Ovarian and oviduct dysfunction may lead to abdominal ovulation or oviduct impaction, which, in turn, may bring about extensive peritonitis and adhesions of the abdominal viscera. Ascites may also develop. Quite frequently, pericarditis is observed in both females and males. The changes that occur in the pericardium, epicardium and pericardial fluid appear to be dependent on the age of the disease process. In acute cases, the pericardium exhibits only a slight translucency, and the pericardial fluid may be increased and turbid. In more advanced stages, the pericardial sac is thickened and opaque, and the pericardial fluid is greatly increased in amount containing considerable exudative material. This may be followed by permanent thickening of the pericardium and epicardium and partial obliteration of the percardial cavity by adhesions. The liver is commonly swollen greenish-brown or bronze in colour. There may be microabscesses scattered over the liver and myocardium. The spleen is also normally swollen and may have microabscesses. In the male, the infection is frequently found in the reproductive organs, especially localisation in the testicle (Pomeroy, 1972).

2.2.3 Shedding of the Disease Organisms

The excretion of salmonellae from infected birds has been found to be intermittent (Magwood and Bigland, 1962, Brownell *et.al.*, 1969; Smith *et.al.*, 1972; Brown *et.al.*, 1975). This indicates that, if one does not detect salmonellae with the cloacal-swab method, (cloacal swabbing and culturing), it does not mean that a bird is completely free from salmonellae. One may have swabbed during the non-shedding period. This is explained further by Smith *et.al.* (1972), who dosed 12 birds with *S. virchow* and found that in 2 out of the 12 birds, *S. virchow* was never detected using cloacal swab monitoring. However, serologically, a continuous increase in antibody activity to *S. virchow* was detected in the 2 birds. Similar findings were reported by Brown *et.al.* (1975). They experimentally infected 34 cockerels with *S. typhimurium* and found that in 2 of the cockerels, *S. typhimurium* was never detected using the cloacal-swab method. However, the organism was recovered from the tissues of the 2 cockerels at necropsy. In a survey carried out to screen 4 farms and a slaughterhouse, in Kenya, for the occurrence of Salmonella infections, done mainly by cloacal swabbing and serology, it was found that cloacal swabbing and culturing gave an indication of only 0.4% occurrence rate in the birds surveyed, while serology of the same birds indicated an occurrence rate of 37.8% (Bebora *et.al.* 1979). However, some workers have had success in isolating salmonellae from cloacal swabs (Faddoul and Fellows, 1966; Kumar *et.al*, 1971 b).

The shedding of salmonellae in birds is influenced by stress like muscular fatigue, cold, heat, wetness, limitation of food and water and concurrent infection (Brownell *et.al.*, 1969). Brown *et.al* (1975) found that cloacal excretion of *S. typhimurium* in cockerels occurred during the first 5 days of infection, after which the excretion rate dropped considerably. Nyaga *et.al.*, (1981) working on Marabou storks, observed that excretion of *S. typhimurium* started as early as 3 hours after dosing the birds with 20 mls. of a 2.0 x 10^9 org/ml. suspension. Intermittent excretion of salmonellae could be explained by the fact that *Salmonella* bacteria are capable of residing within macrophages (Campbell, 1976). These intracellularly located salmonellae may be extracted from the macrophages only during stress or at periods when the organisms have multiplied to very large numbers that the macrophages are lysed. The fact that *Salmonella* serotypes react differently to various culture media (Harvey and Price, 1975) should be taken into account, here, because some of the failures to isolate the organism may be due to wrong usage of the media.

In intestinal infections, the organisms seem to have a predilection for the caeca and caecal tonsils (Brownell *et.al.*, 1969; 1970; Fanelli *et.al.*, 1971; Turnbull and Snoeyenbos, 1974; and Brown *et.al.*, 1975). Brownell *et.al* (1970) reported that birds whose caeca were surgically removed or ligated had a higher shedding rate than the controls. They did not find any evidence in chickens that the caecum plays an essential role in establishing or maintaining intestinal infection with *S. typhimurium*. However, it is possible that it may serve as a mechanical localisation site for salmonellae introduced into the intestinal tract. Following oral inoculation, the crop also appeared to be a potential reservoir of persisting infection at all ages (Turnbull and Snoeyenbos, 1974).

2.2.4 Transmission of the Disease

Salmonella organisms can be transmitted in a number of ways. Egg-transmission as a result of localisation of the organisms in the ovaries has been reported in *S. gallinarum/S.pullorum* infections (Snoeyenbos, 1972; Pomeroy, 1972); as well as transmission through egg-shell penetration (Williams and Whittemore, 1967; Williams and Dillard, 1968 a, b; 1969; Williams *et.al.*, 1968; Chowdhury *et.al.*, 1976). Since the most common sign of this disease is enteritis (Pomeroy, 1972), the excreta of an infected flock is very infective. This contaminates feed and/or water and results in transmission of infective organisms to susceptible birds through the oral route. Gwatkin and Mitchell (1944) found that the organisms could be transmitted by flies. Transmission through inhalation has also been reported (Bunyea and Hall, 1929).

Transmission could be from wild birds which hover over poultry houses. This possibility has been confirmed by the work of Nyaga *et.al.* (1981) who worked on experimental infection of Marabou storks with *Salmonella typhimurium*. The birds were dosed individually *per os* with 20 millilitres of a bacterial suspension containing 2.0×10^9 organisms per millilitre of *S. typhimurium* and faecal samples were obtained daily from each of the birds for 5 days and were immediately cultured on MacConkey agar plates for bacterial reisolation. *S. typhimurium* was reisolated from faecal samples as early as 3 hours and as late as 12 days after dosing, which shows that these birds can transmit the disease organisms even though they don't come down with the disease.

2.2.5 Epidemiology

While the chicken appears to be the natural host of *S. gallinarum*, other species have also exhibited some degree of susceptibility especially the turkeys, guinea-fowl and pea-fowl. Mammals are variably susceptible. *S. gallinarum* infections in man have been reported, (Pomeroy, 1972).

2.2.6 Immune Responses to Salmonella

Salmonella infections induce both cell-mediated and humoral immune responses. Of these, cell-mediated immunity has been shown to be the one responsible for protection against the invading organism (Collins, 1972a; 1974; Dannenberg, 1968; Mackaness and Blanden, 1967; Nelson, 1972; Suter and Ramseier, 1964; WHO Scientific group, 1973). *Salmonella* organisms, like *Mycobacterium*, *Brucella*, *Listeria*, can survive within normal macrophages without being degraded (Collins, 1974; WHO scientific group, 1973; North, 1974). They are, thus, referred to as facultatively intracellular organisms. For degradation of the organisms to occur, under normal circumstances, there is fusion between the phagosome (containing the organisms) and the lysosome (containing the disgestive enzymes) (Campbell, 1976). When this occurs, the two lyse and the digestive enzymes digest the invading organisms. With facultatively intracellular organisms, this fusion does not occur (Armstrong and Hart, 1971; Hart *et.al.*, 1972; Jones and Hirsh, 1972). This fusion only occurs in delayed hypersensitivity reaction where the macrophages are activated and hence become verocious (Mackaness, 1970; 1971; Suter and Ramseier, 1964; Campbell, 1976).

When cell-mediated immunity develops in response to infection of these intra cellular bacteria, it is manifested by immune T-cells releasing lymphokines (Macrophage Migration inhibition factor) that activate macrophages. The activated macrophages have increased ability to destroy microorganisms, increased metabolic activity, increased lysosomal enzymes, increased number of lysosomes, and increased ability to spread on glass, *in vitro*, which indicates increased phagocytosis. Thus, the activated macrophages much more effectively phagocytose the bacteria and either kill them or inactivate them. This action of macrophages is non-specific (Zinkernagel, 1976; Viken *et.al.*, 1977; Ashley *et.al.* 1977; Biroum-Noerjasin, 1977). This activation of macrophages is a complex series of structural and biochemical activity. The T-cell also produces other lymphokines like:- (i) lymphocyte blastogenic factor, which stimulates B-lymphocytes to produce blast cells and differentiate into plasma cells and (ii) cytotoxic factor which is used for direct T-cell killing of the target cells.

The activation of macrophages is termed Delayed hypersensitivity reaction (DHS). This acquired resistance persists as long as the parasite survives and/or DHS persists. Antigenically specific acquired immunity and DHS can be recalled rapidly by infection (Ashley *et.al.*, 1977). There is enough evidence in support of the hypothesis that DHS plays a mediator role in cellular immunity. Collins (1971), Collins and Mackaness (1968; 1970), and Mackaness and Blanden (1967) demonstrated that rodents immunised with facultative intracellular parasites, elaborate an immunity which is consistently associated with DHS. Waiyaki (1974) found that maximum DHS to most *Salmonella typhimurium* antigens occurred at about the same time when there was maximum reduction of *Salmonella* organisms in the livers and spleens of infected mice.

Of considerable biological importance, materials which are, or are part of invading bacteria have been reported to stimulate macrophages directly, for example, lipopolysaccharide or its lipid A derivative can activate macrophages (Alexander and Evans, 1971; Cohn and Morse, 1960; Fox and Kausalya, 1980; Landy and Braun, 1964; Morrison and Ryan, 1979). Culture filtrates from *Listeria monocytogenes* cultures also activate macrophages (Petit and Unanue, 1974). BCG organisms, too, have been reported to bind and activate macrophages and to enhance their metabolic activities (Berthrong, 1970; Blanden *et.al.*, 1969; Dannenberg, 1968; Ratzan *et.al.*, 1972). In addition to this, several reports, in the literature, ascribe the macrophages the ability to produce a variety of mediators that can act on other cells. In studies on *in vitro* immune response, the mixed leucocyte reaction, macrophages or a factor secreted by them, have been shown to be required for the reactions to occur (Alter and Bach, 1970, Bach *et.al.*, 1970; Hersh and Harris, 1968). Similarly, production of lymphokines, by T cells responding to antigenic challenge, has been reported to require the presence of viable macrophages (Wahl *et.al.*, 1975; Oppenheim *et.al.*, 1968; Alter and Bach, 1970). It has also been suggested that macrophages are required for generation of T-helper-cells' activity *in vitro*.

The specificity of the response is, however, vested in the lymphocytes (Levis and Robbins, 1970; Hanifin and Cline, 1970). Macrophages may act by concentrating antigen at their surface (Cline and Swett, 1968; Hanifin and Cline, 1970) where it can more effectively stimulate lymphocytes. A soluble factor elaborated by cultured normal macrophages has, however, recently been described as capable of restoring activity to pure lymphocyte populations (Nelson, 1972).

Macrophages appear to be able to elaborate factors that are directly bactericidal. *Listeria* cells were killed by products of macrophages incubated with stimulated lymphocytes (Bast *et.al.*, 1974; Middlebrook *et.al*, 1974). A soluble material released from mouse macrophages immune to *Listeria monocytogenes* that exerts antilisterial activity *in vitro* has been described (Sethi *et.al.*, 1974). In addition guinea pig alveolar macrophages, cultured with PPD, released a cytotoxic factor (Heise and Weiser, 1969). Youmans and Youmans (1962 a, b) extracted from the lungs of BCG-immunised, but not from normal, rabbits and guinea pigs a factor which they termed "mycosuppressin"; this factor inhibited the growth of mycobacteria *in vitro*. Ramseier and Suter (1964 a, b) extracted a factor, which had antimycobacterial activity *in vitro*, from the nuclei of peritoneal cells of guinea pigs, both normal and BCG-vaccinated. Gershon and Olitzki (1965) extracted from macrophages a basic protein, "monocytin", with more general antibacterial activity *in vitro*.

Recent evidence demonstrates that B-cells can also produce lymphokines which are indistinguishable from those of T-cells (Yoshida *et.al.*, 1973; Florentin *et.al.*, 1975; Rocklin *et.al.*, 1974; Bloom *et.al.*, 1975; Subba-Rao and Glick, 1977). Bloom *et.al.*, (1975) believed that it is possible to have a very small number of residual T-cells in the purified B-cell population, which may produce the lymphokines. However, Subba-Rao and Glick (1977) did not have supportive evidence for this possibility, in their experiment, since their findings demonstrated different thresholds of response for thymus and bursal cells in chickens. Bursal cells peaked in what they called Lymphocyte inhibition factor (Lylf) production by 5 weeks of age, whereas the thymus cell production of LyIF, at that time, was minimal. Thymus cells did not attain their peak LyIF-production until 12 weeks of age and, at that time, bursal cell LyIF production was declining. *In vivo* evidence also shows that B-cells are involved in cell-mediated immunity. Lethally irradiated mice, injected with normal, isologous bone-marrow cells could resist challenge by *Listeria monocytogenes* (Campbell *et.al*, 1974).

The role of antibody in *Salmonella* infections has been a centre of controversy for a number of years. Collins (1971) claims that cellular immunity is the main protective factor and Jenkins and Rowley (1965) and Rowley *et.al.*, (1968) have argued that immunity to salmonellosis is humoral in nature. Their studies showed that specific opsonins could be sufficient in protection against lethal salmonellosis. They also cited the finding that in field trials involving use of killed vaccines (Yugoslav Typhoid Commission, 1972), specific antibody protected against typhoid fever. This idea was weighted on by findings of Ornellas *et.al.*, (1970), Herzberg *et.al.*, (1972), Kenny and Herzberg (1968), Badakhsh and Herzberg (1969), Smith and Bigley (1972 b), who worked on different types of dead vaccines. Cameron and Fuls (1976 a, b) compared live and dead vaccines given parenterally and found that both can be used to immunise successfully against infection with *S. dublin*. However, Meyer *et.al.*, (1977) found that live antigen of *S. dublin* gave better results than heat-treated dead antigen when administered orally for prevention of *S. dublin* infection of calves. Similar results were reported by Collins and Carter (1972) and Smith (1956 a, b).

Cameron (1976) found that, although cellular immunity is a prominent factor in protection of *Salmonella*, cross-protection between two serotypes was not appreciable.

This same finding was reported by Davies and Kotlarski (1976), when they found that elimination of *S. typhimurium* from mice previously immunised by *S. enteritidis* was not as rapid as in mice previously immunised by *S. typhimurium* and they related this to absence of antibody against *S. typhimurium.* This was in agreement with Collins' (1968) findings that elimination of homologous challenge dose was faster than of heterologous one. Collins concluded that there was an element of specificity in the immunity expressed against the two salmonellae and suggested that the two strains were not identical with respect to the antigens involved in the activation of cellular mechanism(s) responsible for bacterial inactivation. However, Davies and Kotlarski (1976) suggest that it may be the lack of antibody specific for the heterologous strain of *Salmonella* that limited the expression of cellular immunity to the heterologous challenge. These are the cytophilic antibodies as suggested by Hoy and Nelson (1969), Granger and Weiser (1964, 1966), and Granger *et.al.*,(1966). These cytophilic antibodies are carried on the macrophages and they are normally IgM. So, in all the situations in which macrophages exert a specific cytotoxic or suppressive effect on target cells in the absence of free serum, the specificity seems most likely to be due to cytophilic antibodies carried on the macrophages. These antibodies seem to be responsible for the initial contact between macrophages and target cells.

Macrophages treated with trypsin lost their capacity to adhere to target cells and destroy them. They regained this capacity after exposure to immune serum. Normal macrophages exposed to immune serum did not acquire this capacity. It is interesting to note, here, that antibodies to *Salmonella* have been detected on macrophages from immunised mice (Rowley *et.al.*, 1964; Kurashige *et.al.*, 1967).

The role of antibody to *Salmonella* protection has also been suggested by Hochadel and Keller (1977) who worked on transfers of T & B lymphocytes from mice known to be immune to *S. typhimurium* to normal mice and then challenging the recipients with lethal doses of *S. typhimurium*. They suggested that failure of other workers (Jenkins and Rowley, 1963; Collins, 1969 a) to appreciate the role of antibody is because they transferred hyperimmune sera, where the half-life of the immunoglobulins is very short, hence did not persist long enough to affect the outcome of the infection measurably. Passive transfer of B-lymphocytes is a more effective way to ensure high levels of antibodies as long as the infecting organisms are present.

2.2.7 Age and Immune Response

Investigators generally accept the fact that immunoglobulins demonstrable by serological test procedures in avian species are stimulated more readily and reach higher levels as birds mature (Wolfe *et.al.*, 1957). Although we know that the chicken embryo is capable of an immunologic response during the second half of embryogenesis (Van Alten and Schechtman, 1963), newly synthesised antibodies may not be evident until several weeks after hatching (Buxton, 1954; Fox and Laemmert, 1947; Kramer, 1973). Wolfe and Dilks (1948) demonstrated that newly-hatched chicks failed to respond or responded very weakly to a parenteral injection of bovine serum. The percentage of birds yielding detectable precipitins increased through the first 4 weeks but increased suddenly and to a rather large extent between 4th and 5th weeks. Wolfe *et.al.*, (1957) administered bovine serum albumin intravenously to chickens and found statistically significant increases in precipitin productivity between 6 and 12 weeks and between 12 and 22 weeks. Serological maturity was reached between 22 and 25 weeks. Buxton (1954) found that inoculation of living *Salmonella pullorum* cells into newly hatched chicks resulted in antibody production that was demonstrable only 11 to 30 days after inoculation. Hirata and Schechtman (1960) reported that chickens do not attain full capacity for antibody formation until they are about 22 weeks old. However, Williams and

Whittemore (1975) found that birds 6 weeks old and older responded well with high antibody titres when infected orally with *S. typhimurium.*

Present regulations of the National Poultry Improvement Plan (Anon. 1975) require that chickens and turkeys be at least four months old before they are serologically tested for *Salmonella* infections. Kumar *et.al.*, (1971 a) reported that turkeys infected with *Salmonella typhimurium* at four weeks old had higher agglutination titres than turkeys infected at one-day old, and also that the percentage of birds serologically positive was higher. Turnbull and Snoeyenbos (1974) were unable to detect significant antibody levels in chickens inoculated with *Salmonella enteritidis* at 1 or 14 days of age. Antibodies were detected at 9 and 18 days after inoculation of adult birds.

2.2.8 Diagnosis of the Disease

Diagnosis of salmonellosis can be based on three aspects namely:- (i) Bacteriological examination of the organisms, (ii) tests that will detect humoral immunity, and (iii) tests that will detect cell mediated immunity. The bacterial examination, in living birds, is normally done by cloacal swabbing and culture using the methods given earlier under "isolation and identification".

If the birds are dead, the contents of the intestinal tract are frequently used for detecting salmonellosis. One can also culture these organisms from the livers and spleens of the infected birds. In practice, the organisms can be recovered from any affected organ including the joints, in cases of arthritis.

Serological tests detect humoral immunity. These are normally carried out to detect the presence of antibodies to *Salmonella* organisms. They include:- slide agglutination test (SAT) or rapid serum agglutination test (RSPT), tube agglutination test (TAT), rapid whole blood plate test (RWBPT), microagglutination test (MAT), macro-or micro-antiglobulin test (MAGT), indirect haemagglutination test (IHA) etc. The use of TAT was reported as early as 1913 (Jones, 1913; Gage *et.al.*, 1914; Rettger *et.al.*, 1915). RWBPT was developed by Schaffer *et.al.*, (1931) while RSPT was developed by Runnells *et.al.*, (1927). MAT and micro-MAGT were described by Williams and Whittemore (1971 and 1972, respectively), while MAGT was practised as early as 1908 (cited by Coombs and Stoker, 1951). IHA was reported by Neter *et.al.*, (1952a).

TAT has been found to be more sensitive than RWBPT and SAT (Bebora *et.al.*, 1979; Williams and Whittemore 1976 b). This is to be expected since SAT and RWBPT require a higher serum antibody titre to give positive results

(Smith *et.al.*, 1972). They are read within 2 minutes, while TAT has a longer period to act and the reagents are incubated at 37°C, optimal temperature for antibody-antigen reaction. RWBPT has been shown to be more sensitive than SAT (Bebora *et.al.*, 1979) and this could be attributed to the staining of the antigen used for RWBPT since that for SAT is not stained. It is easier to view stained substances, especially if small, than to view unstained substances. TAT shows same sensitivity as MAT, although the latter saves time and space (Williams and Whittemore, 1971).

Although RWBPT is widely used for the detection of antibody activity to *S. gallinarum* infections, a case where RWBPT gave negative results when IHA titres were significant and salmonellae were isolated from culture of cloacal swabs has been reported (Smith *et. al.*, 1972). Smith *et.al.*, (1972) also observed that the RWBPT first appeared positive on 8th to 11th days post-inoculation and detected positives for about 24 days after which the sensitivity fell considerably. The reduced sensitivity was due to low antibody titres. Similar findings were reported by Bebora (1979) who found that birds from farms suffering from active fowl typhoid infection gave positive RWBPT reactions, while those with no active infection were mostly negative. Some of these negative birds, however, gave positive results with TAT and MAGT. This shows that

RWBPT is unreliable in cases where the antibody titre is low.

The ability of TAT to dependably detect infected chickens has been questioned (Yamamoto *et.al.*, 1962; Olesiuk *et.al* 1969) since it may not be able to detect intestinal infections. It is common to find antibody titres of 20, to 'O' *Salmonella* antigen in known negative birds (Smith *et.al.*, 1972). *Salmonella* antibody titres between 40 and 80 have been reported in normal cattle (Hall *et.al.*, 1978; Hinton, 1973; Field, 1948; Clarenburg and Vink, 1949). It should however, be noted that agglutinating antibody titre in the blood tends to fluctuate considerably (Gordon and Garside, 1944; Gordon and Buxton, 1945; Karlshoj and Szabo, 1949; Blaxland and Blowers, 1951). Thus, negative agglutination test on a flock cannot be regarded as conclusive evidence of freedom from infection. Sera, could also show negative antibody activity with TAT as a result of presence of "incomplete" antibodies (Coombs and Stoker, 1951).

MAGT detects more positives and gives higher titres than TAT (Bebora *et.al.*, 1979; Coombs and Stoker, 1951; Williams and Whittemore, 1972; 1976 a, b). Coombs and Stoker (1951), working on Q-fever antibody detections found that MAGT could detect titres as high as 64 times those of TAT, and majority of patients which showed no antibody activity with TAT showed titres up to 160 with MAGT. Bebora *et.al.*, (1979) had one serum sample which did not show any *Salmonella* antibody activity with TAT but had MAGT titre of 20,480. This was rather high and could only be possible if the bird in question had abnormal catabolism of antibodies whereby there were many univalent antibody fragments or the animal could be abnormally producing many univalent antibody fragments. The possibility of cross-reactions with other members of *Enterobacteriaceae* could explain some of the high antibody titres detected in MAGT. When a preliminary experiment was carried out screening cross-reactivity of *Salmonella* polyvalent "O" antiserum with *Escherichia*, *Proteus*, *Aerobacter*, *Citrobacter* and *Pseudomonas*, it was found that there was strong cross-reaction with *Escherichia*, *Proteus*, *Aerobacter* and *Citrobacter* but not with *Pseudomonas* (Bebora, 1979). Cowan (1974) reported the presence of common enterobacterial antigens. This could explain the cross-reactivity since it occurred strictly between organisms of the family *Enterobacteriaceae*. Organisms not in the family, for example *Pseudomonas*, did not agglutinate with the antiserum.

Wray *et.al.*, (1975) compared IHA and TAT for serological diagnosis of *Salmonella dublin* infection in cattle and found that, generally, IHA was more sensitive and gave higher titres than TAT. However, IHA occasionally detected serological activity at high titre in cattle with no history of *S. dublin* infection. In some cattle which were positive for *S. dublin* on culture, neither TAT nor IHA tests detected diagnostic levels of antibodies. IHA has also been reported as being more sensitive than TAT by other workers (Neter *et.al.*, 1954; Smith *et.al*, 1972; Carrere and Roux, 1952). Smith *et.al.*, (1972) has reported successful application of IHA in detection of avian salmonellosis. Other tests such as the precipitin test and the complement fixation test were found either unreliable or impracticable in the detection of carriers (Edwards and Hull, 1929).

wattle test *

Two tests are commonly used to detect cell-mediated immunity, namely:- (i) skin test or wattle test, and (ii) macrophage migration inhibition test. The wattle test is done by injecting the antigen subcutaneously or intradermally into one wattle of a chicken. If the bird has cell-mediated immunity or delayed-type hypersensitivity (DTH) to that antigen (positive reaction), there will be swelling in the injected wattle. The thickness of the wattles are measured in order to give the degree of swelling. Routinely, this measurement is done twice i.e. 24 hours and 48 hours post-injection. Care should be taken, here, since some of the swelling may be due to Arthus reaction and not DTH (Collins and Mackaness, 1968). Wattle test (WT) was first applied to detect carriers of pullorum disease and was first reported by Ward and Gallagher (1917). Later it was extensively investigated by other workers (Bushnell and Brandly, 1929; Edwards and Hull, 1929; Michael and Beach, 1929; Rettger *et.al.*, 1930). Their findings showed that the method was not sufficiently reliable for practical use in the detection of carriers. However, Stefanov *et.al.*, (1974) compared WT with the blood agglutination test and found that WT, read 24 hours after injection of allergen, detected more infected birds than the blood agglutination test. Timms and Cullen (1976) showed that skin test is a less sensitive indicator of cell-mediated immunity than the macrophage migration inhibition test.

The macrophage migration inhibition test (MIT) is well known and widely studied as an *in vitro* correlate of delayed-type hypersensitivity (Bloom and Bennett, 1970; David, 1968). Analysis of the reaction has shown that it is usually mediated by a lymphokine (Migration inhibition factor, MIF) produced by committed lymphocytes reacting with antigen. In most studies MIF has been found to act independently of antigen but, in some experiments, this product has been found to inhibit migration only, or much more markedly, in the presence of antigen (Svejcar *et.al.*, 1968; Bennett and Bloom, 1967; Amos and Lachmann, 1970). The test involves harvesting of leucocytes which include both lymphocytes and macrophages and polymorphonuclear leucocytes. These are filled into non-heparinised capillary tubes and centrifuged. The capillary tubes are cut at the cell-fluid interface and mounted in Sykes-Moore chambers. It is always done in duplicates:- to one chamber is added minimum essential medium (containing antibiotics) only; to the other chamber is added the medium plus antigen. The chambers are incubated for 18 hours and migration areas measured. The percent migration inhibition is then calculated using the formula:-

$$\left(1-\frac{\text{migration area with antigen}}{\text{migration area without antigen}}\right) \times 100$$

It is safer to run more chambers for each antigen and then take the average migration area.

The leucocytes could be harvested from three sources, namely:- (i) the buffy coat (Falk and Zabriskie, 1971; Belsheim, 1981), (ii) the peritoneum (Morley *et.al.*, 1973; Granger *et.al.*, 1970), and (iii) the spleen, thymus or lymphnodes (Falk and Zabriskie, 1971; Adler *et.al.*, 1970). If leucocytes are to be harvested from the buffy coat, the blood is collected into heparinised Dulbecco phosphate buffer. This is then centrifuged and the buffy coat fraction aspirated. The cells are washed three times with phosphate buffered saline (PBS). The cells are then counted and made to a concentration of 2 x 10^7 cells/ml. There may be red-cell contamination but it has been found that contamination to the ratio of 1:1 does not affect the migration pattern (Timms and Cullen, 1976). Timms and Cullen (1976) showed that leucocyte migrations free from red cells gave a less distinct image on projection, making the test harder to read.

The leucocytes can be induced to concentrate in the peritoneal cavity by injecting sterile liquid paraffin oil (20-30 ml) intraperitoneally (Morley *et.al.*, 1973), or by injecting hydrolysed starch (Granger *et.al.*, 1970). The peritoneal cells (containing 70% macrophages, 20% lymphocytes) are collected aseptically by peritoneal irrigation with 100 ml. quantities of cold Hank's balanced salt solution, ph7.2. The suspension is then centrifuged and the oily Hank's medium aspirated. The cell-pellet is washed twice with 10-ml. volumes of cold Hank's solution by centrifugation and the pellet is then resuspended in 20 volumes (1 to 2ml.) of cold

Eagle's minimum essential medium at pH7.5. Following a cell-count, the volume of the cell suspension is adjusted to 1.6 x 10^7 cells/ml. The spleen, thymus or lymphnodes can be removed aseptically. Each organ is gently minced with scissors and the tissue fragments screened through 60-mesh stainless steel gauze into the complete tissue culture medium. The cells are drawn through a 25-gauge needle to produce a suspension of single cells. The cells are then washed, counted and adjusted to 4 x 10^7 cells/ml.

The MIT can be run using capillary tubes as given above (Falk and Zabriskie, 1971; Morley *et.al.*, 1973) or by use of a modified migration under agarose technique (LMAT) (Belsheim, 1981). When done using capillary tubes, after 15-18 hours incubation, areas of cell-migration are determined by planimetry with the aid of a projection microscope which produces enlarged image of the cells (Falk and Zabriskie, 1971). Alternatively, migration areas may be measured by weighing photographed or traced *camera lucida* images on paper of uniform texture. At any given concentration of lymphokine in the culture chamber, the mean area of cell migration is expressed as a ratio of the area of the cell migration into tissue culture medium with and without antigen.

This quotient, termed the "migration index" is taken as the parameter of response on which assays of migration inhibition activity can be based. Morley *et.al.*, (1973) suggest an incubation period of 15-18 hours. This is because inhibition of macrophage migration by lymphocytes is a temporary phenomenon and they found that if macrophage cultures were incubated for 24 hours, the migration "front" of cells was sometimes less well-defined than at 15-18 hours. It has been found that the results will be the same whether the cells are harvested from the buffy coat, peritoneum or tissues (Solotorovsky and Soderberg, 1972).

Microscopic examination of leucocyte preparations and migration areas show that inhibition of migration involves the participation of both lymphocytes, macrophages and polymorphonuclear leucocytes (Timms and Cullen, 1976; Timms, 1974). Evidence of participation of polymorphonuclear leucocytes in MIT has been provided by Søborg (1969) and Clausen (1970) who have shown that antigen-induced inhibition of human peripheral leucocytes occurs only when both sensitised lymphocytes and granulocytes (which do not need to be sensitised) are present. Strastry and Ziff (1970) also described an inhibitor of macrophage migration which appeared to be derived from polymorphonuclear leucocytes. It can be concluded, therefore, that in these present studies, the sensitive lymphocyte is the immunologically specific cell and the granulocytes are acting as indicator cells in the micro-environment of the chamber to demonstrate inhibition of the whole buffy coat population, consisting of lymphocytes, macrophages and granulocytes. It is possible that granulocytes are also producing an inhibitory substance which is acting upon the migration of macrophages. Dumonde and Maini (1971) affirm that soluble antigen-antibody complexes can exert a range of biological effects, such as inhibition of macrophage and polymorph migration, which bear some relation to those of cellular immune mediators or lymphokines. The MIT reported in these papers may also be the result of co-existing humoral immune mechanisms as well as lymphokine factors.

The MIT has proved to be a good *in vitro* indicator of cell-mediated immunity although percent migration inhibitions of 9-11% have been found in known negative birds (Timms and Alexander, 1977). Those who have compared MIT with Wattle test have shown no correlation between magnitude of the skin test and positive values with MIT (Timms, 1979). The skin test has been shown to be a less sensitive indicator of cell mediated immunity than MIT (Timms and Cullen, 1976).

Due to the unreliability of the wattle test (Bushnell and Brandly, 1929; Collins and Mackaness, 1968) and the involvement of the MIT (Morley *et.al.*, 1973), workers have resorted to screening of birds bacteriologically and/or serologically (Williams, 1978). Of these two, serological diagnosis has proved to be more sensitive and reliable. The serological tests rank from the most sensitive to the least sensitive as follows:- MAGT, IHA, TAT (MAT), RWBPT, SAT (RSPT) (Williams and Whittemore, 1976 b). The cloacal swab method is inadequate for *Salmonella* detection in chickens (Williams and Whittemore 1976 b, Bebora *et.al.* 1979). This is because faecal excretion of the organisms is variable (Magwood and Bigland, 1962; Brownell *et.al.*, 1969; Smith *et.al.*, 1972; Brown *et.al.*, 1975). This may be rectified by culturing three cloacal-swab samples rather than one from each bird (Annual report, 1967). Williams and Whittemore (1975) found that positive cloacal swabs tended to decline rapidly in all birds tested after oral infection with *S. typhimurium*. However, for actively infected birds, *Salmonella* isolation from intestinal tract has proved to be reliable (Faddoul and Fellows, 1966; Brownell *et.al.*, 1969).

In an effort to reduce time taken with culture methods, several investigators have developed fluorescent antibody methods for foods and faecal specimens (Georgala and Boothroyd,1964; Haglund *et.al.*, 1964). Where conjugated antisera against somatic antigens only have been used (Georgala and Boothroyd, 1964; Lovelock and Davies, 1966) cross-reactions with other bacteria have been a problem often necessitating extensive absorption of antisera. However, where conjugated antisera against flagellar antigens have been used (Haglund *et.al.*, 1964; Caldwell *et.al.*, 1966; Ellis and Harrington, 1968) this problem has been reduced. Moreover, Svenungsson and Lindberg (1979) showed high specificity when they used conjugated antiserum specific for *Salmonella* O-antigen 8 in indirect immunofluorescence test.

2.2.9 Treatment and Control

Outbreaks of fowl typhoid have been successfully treated by a number of antibiotics like furazolidone, neomycin, neolidone and chloramphenicol (Poultry Clinic, University of Nairobi, Unpublished data). In order to pick one that is effective for the particular outbreak, antibiotic sensitivity testing is normally carried out for each isolate per outbreak. The treatment does not necessarily get rid of carriers. These serve as a constant source of the disease organisms (Pomeroy, 1972).

Therefore, a number of countries have resorted to blood testing and eradication programmes for fowl typhoid/ pullorum disease (Pomeroy, 1972; Schwartz, 1972). The blood-testing is done using RWBPT, and all positive reactors or the whole flock is killed and the birds disposed. The houses are then thoroughly disinfected, preferably followed by fumigation with formalin, before any other birds are brought in (Blaxland *et.al.*, 1958; Robinson, 1970; Snoeyenbos, 1972; Schwartz, 1972). Where eradication cannot be practised, hygiene must be practised to the maximum, to make sure the disease is not transmitted to the other "clean" birds. Hatchery and flock sanitation is most important in prevention and control of the disease. Adult intestinal carriers are the main source of infection in poultry.

The most common way of preventing the disease is by use of vaccines. Vaccination is used the World over and has shown considerable success in preventing the disease (Pomeroy, 1972).

2.3 VACCINES AND VACCINATION

A number of workers have shown that, when assessment of protection is made on the basis of survival rates, immunisation with killed *Salmonella* vaccine, which includes a significant humoral response, or passive transfer of antibody specific for a particular strain of *Salmonella*, will protect mice against intraperitoneal challenge with the same strain of *Salmonella* (Jenkins and Rowley, 1963; Venneman and Berry, 1971; Herzberg *et.al.*, 1972). It has also been shown that if control of multiplication of the challenge organism is used as the criterion for immunity, effective protection of mice can only be achieved using living and not killed *Salmonella* vaccines (Collins *et.al.*, 1966; Collins, 1969 a, b). This is because inactivation of challenge organisms requires the presence of activated macrophages which are only induced by live vaccines (Mackaness, 1970).

There have been various vaccines formulated in the effort of producing immunity to *Salmonella* organisms. Dead vaccines have been used widely, and a number of investigators claim success in protection (Jenkins and Rowley, 1965; Rowley *et.al.*, 1968; Ornellas *et.al.*, 1970; Herzberg *et.al.*, 1972; Kenny and Herzberg, 1968; Badakhsh and Herzberg, 1969; Waldman *et.al.*, 1972; Bairey, 1978). However from what has been argued earlier on the immunity to *Salmonella*, it can be concluded that killed vaccines are of little value in the control of salmonellosis. This is because the killed vaccines give rise to humoral immunity only and not to cell-mediated immunity (Davies and Kotlarski, 1976; Solotorovsky and Soderberg, 1972). The protective effect of specific antibodies is based on their ability to reduce the size of initial viable challenge inoculum rather than any discernible influence on the subsequent rate of multiplication of parasites (Collins, 1969 a, b; 1971; 1974; Solotorovsky and Soderberg, 1972). Indeed, Diena *et.al.*, (1977) found that there was no significant difference in protective ability between live and acetone-treated typhoid vaccine when given intraperitoneally, while the live vaccine was significantly more protective than the killed one when given orally. Killed vaccines can be produced in a number of ways e.g. by heating and by treatment with formalin, acetone, alcohol or deoxycholate. Diena *et.al.*, (1973) have reported that acetone-treated vaccines were markedly more protective than the heat-killed, phenol-preserved vaccines.

A live, avirulent, stable, rough strain (9R) of *S. gallinarum* has been used extensively to protect poultry against fowl typhoid (Smith 1956 b; Arda, 1971; Gupta and Mallick, 1976 a, b). This vaccine could be used subcutaneously (Silva *et.al.*, 1981) or orally, preceded by sodium bicarbonate treatment to neutralise the acid in the contents of proventriculus and gizzard (Gupta and Mallick, 1976 a, Arda, 1971); and could be used either from broth culture (Gordon *et.al.*, 1959;

Gordon and Luke, 1959; Harbourne, 1957; Smith, 1956 b) or from a reconstituted freeze-dried state (Harbourne *et.al.*, 1963, Smith, 1969). It was found that while the oral *S. gallinarum* vaccination showed protection to oral challenge (Gupta and Mallick, 1976 a; Arda, 1971), the subcutaneous vaccination gave variable protection to chickens, against both natural and experimental intestinal fowl typhoid (Silva *et.al.*, 1981, Harbourne *et.al.*, 1963; Smith, 1956 b; 1969). It, however, gave good protection against parenteral challenge. Better immunity was observed at 8 weeks of age than at 4 weeks (Gordon *et.al.*, 1959) and has not been shown to affect egg-production (Smith, 1956 b). One of the greatest advantages reported to the use of 9R vaccines is that it does not interfere with tests used for pullorum-typhoid control because it does not produce agglutinating antibody titres (Smith, 1956 b). Freund's complete adjuvant has been shown to enhance the effectiveness of this vaccine (Gupta and Mallick, 1976 b ; Padmanaban and Mittal, 1981).

Live, avirulent, smooth vaccines have been used by a number of investigators (Collins, 1971; Cameron and Fuls, 1976 a, b; Meyer *et.al.*, 1977; Collins and Carter, 1972; Smith, 1956 a, b) who reported success in protection against disease. This protection was assessed by challenging the animals with virulent organism and then monitoring death and/or liver and spleen counts of the challenge organism. Winmill (1961) found that the smooth variant of *S. gallinarum* strain 1909 conferred better protection than the rough variant.

Fractions of bacteria can also be used as vaccines. Svenson and Lindberg (1981) used *S. typhimurium* O-antigen-specific oligosaccharide-protein conjugates and showed that they protected mice against intraperitoneal challenge with 100 times the 50% lethal dose of *S. typhimurium* SH 2201 (04, 12) but not against challenge with *S. enteritidis* SH 2204 (09, 12). The antibodies elicited by the immune response of the saccharide-protein conjugates compared well with those of heat-killed bacteria. Success in protectiveness has also been reported in usage of ribonucleic acid-protein fractions as vaccine (Smith and Bigley, 1972 a; Misfeld and Johnson, 1978; Pepper *et.al.*, 1976, Waiyaki, 1974).

Streptomycin-dependent vaccines have been developed from a number of bacteria, namely:- *Brucella melitensis* (Elberg, 1981; Alton and Elberg, 1967), *Brucella abortus* (Olitzki and Szenberg, 1952), *Escherichia coli* (Linde *et.al.*, 1972; Felsenfeld *et.al.*, 1972), *Salmonella typhi*

(Dupont *et.al.*, 1971 a; Reitman, 1967),*Salmonella typhimurium* (Vladoianu and Dubini, 1975), *Vibrio cholerae* (Felsenfeld *et.al.*, 1970), and *Pasteurella multocida* (Wei and Carter, 1978). From the reports, these have shown protective ability. However, none has been developed from *S. gallinarum*.

Shneitz *et.al.*, (1981) found that anaerobically-cultured caecal/intestinal or faecal contents (mixed broth culture) of adult fowls protected chickens from oral *Salmonella* infections. This is due to competitive exclusion of salmonellae by the heavy flora that develops in the gut. Similar findings have been reported by Rigby and Pettit (1980), Snoeyenbos *et.al.* (1978) and Seuna *et.al.*, (1978).

In Kenya, vaccination against Kikuyu Fowl Disease (Fowl Typhoid) was first reported in the Annual Report of 1917-1918. The results were beneficial and the vaccine was described as a living culture of the causal organism. Three injections were given at 10-day intervals; a culture about six months old was used for the first dose, a three-month old culture for the second and freshly isolated culture for the third. Then, an attenuated strain of *S. gallinarum* was discovered and this was used as a vaccine for more than two decades, until 1960. The origin of this strain was a culture of *S. gallinarum* which was discovered in a cupboard months later. Subcultures from it were found to be avirulent but conferred immunity against challenge by freshly isolated strains of the organism. Seed cultures for vaccine production were not lyophilised but were maintained by monthly subculture. In addition to this attenuated strain, smaller quantities of a formalinised whole culture (Ngong strain) were used in the control of outbreaks on farms distant from the laboratory to which live vaccine could not be delivered easily in viable form (Winmill, 1961).

In 1958, there was a breakdown of the vaccine-both the dead and the live-attenuated vaccines showed little protection. Comprehensive tests of the attenuated product showed that it had lost most of its immunising potency as a result of repeated subculture over years of continuous production. Thus, a new strain had to be adopted. This new strain was 1909S and was attenuated by 23 serial subcultivations on MacConkey agar at 2 day intervals and incubator temperature of 42°C. The vaccine was finally prepared by cultivating 1909S on nutrient agar for 18-24 hours at 37°C. The resulting growth was washed off and standardised to

3,200 million organisms per ml. dose. The vaccine was not freeze-dried but used within 24 hours of production (Winmill, 1961). This is the vaccine used today in Kenya. It is now referred to as CN 180.

This vaccine was found to give demonstrable antibodies in the agglutination test which began to wane off at 22 weeks, and at 26 weeks post-vaccination, it gave negative results (Winmill, 1961). The protective effect of the vaccine was monitored by survival in an infected farm. The vaccine is avirulent to adults and day-old chicks. Birds in full lay were vaccinated and there was no drop in egg-production. However, for the first 3 weeks after vaccination the incubated eggs produced chicks that were infected with *S. gallinarum.* Thereafter, the chicks were "clean". Owners of breeding stock who vaccinate laying fowls with 1909S are thus advised to avoid the incubation of eggs for six weeks after vaccination (Winmill, 1961).

The inactivated vaccine (Ngong Strain) is still in use up to today, for remote areas. It is prepared by formalisation of a virulent smooth strain of *S. gallinarum* which has been grown on nutrient agar. As the other observers have reported, immunity conferred by this vaccine is of a lower order than that given by the 1909S strain (Winmill, 1961).

Routinely, in Kenya, vaccination is done only once-at 8 weeks of age. This is because it is desired that the agglutinating antibody titre be low enough to give negative TAT results, by laying time i.e. about 20 weeks of age. Normally, birds are routinely screened for fowl-typhoid just before lay, using RWBPT. Thus, vaccinal interference of the screening is kept minimal.

3. MATERIALS AND METHODS

3.1 SOURCE OF BIRDS

The chicks were bought at one-day of age from Muguku Poultry Farm, Kikuyu. Their breed was shaver starcross.

The older birds were raised within the premises to the ages of 8 weeks and above as indicated in the text.

3.2 ISOLATION AND CHARACTERISATION OF S. GALLINARUM ISOLATES

The *S. gallinarum* organisms were isolated at the Veterinary Research Laboratories, Kabete, and at the faculty of Veterinary Medicine, University of Nairobi, Kabete, from birds that were either sick or dead. The isolation exercise covered a period of 5 years. The isolations were made mainly from the intestinal contents, bone-marrow and liver; the live birds sacrificed by cervical dislocation before opening. The samples were streaked directly onto MacConkey plates, immediately after the birds were opened up. The plates were incubated aerobically at 37°C. The isolates were then characterised to be *S. gallinarum* using the biochemical methods of Cowan and Steel (1974), and Carter (1975). Organisms showing characteristics of *Salmonella* were typed with *Salmonella* polyvalent 'O' antiserum and specific group antisera (Bacto-sera, Difco Laboratories, Detroit, Michigan, U.S.A.) (Kauffmann, 1975).

The isolates were preserved either by lyophilisation or on Dorsett egg medium.

3.3 PREPARATION OF ANTIGENS

Antigens were prepared for the various tests as described below. For the slide agglutination test (SAT) and tube agglutination test (TAT), the antigen was unstained and was prepared using the method given by Williams and Whittemore (1971). Briefly, organisms were subcultured on MacConkey agar and seeded into Roux flasks (French Square bottles) containing sterile nutrient agar and incubated aerobically at 37°C overnight. Harvests of the cultures were made in saline followed by centrifugation at 5,000 x g for 15 minutes at room temperature. The pellet was treated with 20 volumes of 95% ethyl alcohol (analytical grade). This suspension was heated at 60°C for 1 hour, washed 3 times with sterile saline, and the final bacterial suspensions made to a concentration of 1.3×10^9 organisms per ml. using the McFarland nephelometer. The antigen was stored in 5 ml. volumes at +4°C until used.

The soluble antigen for indirect haemagglutination test (IHA) and Macrophage migration inhibition test (MIT) was prepared after the method given by Neter *et.al.* (1952a). Briefly, the relevant 24-hour broth cultures were boiled for 2-2½ hours, centrifuged at 5,000 x g for 15 minutes at room temperature, and the supernatants collected. The supernatants contained the crude antigen which was preserved in thiomersal and used undiluted. The protein content was determined by the method of Lowry *et.al.*, (1951) and ranged from 2.2 - 5.9 mg/ml.

The stained *S. pullorum* antigen for rapid whole blood plate test (RWBPT), prepared by IFFA MERIEUX, London was commercially obtained from E.T. Monks Chemists, Nairobi.

The working dilution of the *Salmonella* antigen for TAT was determined by titrating the antigen. Doubling dilutions were made in 6 tubes ranging from 1/5 to 1/160. To each tube, an equal volume of known polyvalent 'O' *Salmonella* antiserum was added, thus doubling the corresponding dilution. These were mixed and incubated for 24 hours at 37°C. Any agglutination observed was scored following an arbitrary scale of 1+ to 4+. Distinct agglutination was assigned a value of 4+ and weak agglutination a value of 1+. The highest dilution giving an agglutination value of 3+ was taken as the working dilution. This was 1/40.

3.4 PROCESSING OF RED BLOOD CELLS

Sheep red blood cells (rbc) used were either freshly - prepared or formalin - fixed (Herbert, 1973). They were coated with the respective antigen and used in the IHA test. Briefly, blood was obtained from sheep and received into Alsevers solution. This was centrifuged at 750 x g for 10 minutes at room temperature. The red blood cells were then washed 3 times with saline prior to being coated with antigen (after the method of Neter *et.al.*, 1952a). The crude antigen (prepared earlier) was added to the rbc pellet to make a 2.5% rbc suspension. The mixture was then incubated at 37°C for one hour, stirring repeatedly. The rbc's were then washed 3 times with physiological saline and finally made up to 2% suspension. It was stored in 5 ml. volumes at +4°C until used.

3.5 COLLECTION OF SERUM FOR SEROLOGY AND CELLS FOR MIT

Chicken blood was obtained from the wing vein by venipuncture and the blood to be used for serology was collected into a sterile universal bottle and incubated at 37°C for one hour. The blood samples were then centrifuged at 750 x g at room temperature for 10 minutes and each serum sample collected, put in Bijou bottle and stored at - 20°C until used. The blood to be used as the source of leucocytes was collected into a sterile universal bottle containing 15cc of heparinised calcium - magnesium - free Dulbecco phosphate buffer (H-DPB). The heparin was added at the rate of 20 units heparin per one millilitre DPB. About 5cc of blood was added and this resulted in the blood being diluted 4 times.

3.6 PROCEDURES FOR THE TESTS USED

3.6.1 Rapid Whole Blood Plate Test (RWBPT)

The procedure of Snoeyenbos (1972) for the RWBPT was used. Briefly, a drop of blood from the suspected bird was placed on a white tile, to which a drop of the blue-stained *S. pullorum* antigen was added. These were thoroughly mixed and the tile rocked up and down. In positive cases, blue clumps appeared within 2 minutes of testing.

3.6.2 Slide agglutination test (SAT)

SAT was carried out following the method given by Carpenter (1975). A suspension of the unstained antigen, prepared earlier, was used. Briefly, a drop of this antigen suspension was placed on a microscope slide and mixed thoroughly with a drop of the test serum. The slide was then rocked up and down. In positive cases, clumps appeared within 2 minutes ot testing.

3.6.3 Tube agglutination test (TAT)

The procedure for TAT was as given by Carpenter (1975). Doubling dilutions of the test serum were made in test tubes in volumes of 0.5 ml., after which equal volumes of the standardised unstained antigen suspension were added to respective dilutions. The tubes were shaken thoroughly and incubated at 37°C overnight.

Positive reactions gave clumps on shaking while negative reactions showed no clumps. The highest dilution giving definite agglutination was taken as the serum titre.

3.6.4 Indirect haemagglutination test (IHA).

The procedure for IHA was as given by Herbert (1973). Microtitre 'U' plates (Cooke Engineering Co., Alexandria, VA, San Mateo, Calif) were used. Doubling dilutions of the test sera were made in 50 µl.aliquots. Multiple dilutions were possible by use of the Titertek Multidiluting pipette (Flow laboratories, Rockville, Maryland, U.S.A.). With this multidilutor, eight sets of serial dilutions were completed together as illustrated in figure 1a. To these dilutions, equal volumes (50 µl) of coated erythrocytes were added. The plates were shaken and left at room temperature overnight. Reading was as follows:- Positive reaction, i.e. haemagglutination, appeared as a complete carpet of cells covering the bottom of the wells; while negative reaction i.e. no haemagglutination, appeared as a compact button or small ring at centre of the curved bottom. The titre was taken as the highest dilution giving haemagglutination.

Figure 1a: Titertek Multidiluting pipette (Flow laboratories, Rockville, Maryland, U.S.A.) used to complete 8 sets of serial dilutions together.

3.6.5 Macrophage Migration inhibition test (MIT)

The capillary tube technique of the MIT was done according to the method given by Timms (1974). Peripheral blood cells, diluted 1:4, were used as the source of leucocytes. Fifteen (15) mls. of the diluted blood was layered carefully over 4 ml. of Ficoll/Hypaque gradient solution (Nyegaard and Co. A/S Oslo, Norway) to give the required ratio of 8 ml. ficoll/hypaque: 30cc of diluted blood as given by Nyaga (1975). In order to facilitate smooth delivery of the diluted blood onto the gradient surface without disturbing the interface, the walls of each tube were coated with a thin layer of the gradient solution. A small amount of blood was then run gently down the moist tube-walls in a smooth broad band, reaching the gradient surface as a broad fluid front. The rest of the blood was added in a similar manner until all the required volume was run. A distinct interface was formed between the blood and the gradient solution.

The blood was then centrifuged at 1,000 x g for 20 minutes. There was a distinct separation of the buffy coat and the red-blood-cells. The buffy coat was harvested into a sterile Bijou bottle and washed twice with minimal essential medium (MEM) to which was added 100 units of penicillin, 100 mg of streptomycin and

15% foetal bovine serum as given by Timms (1974). The pH of the growth medium ranged from 7.2 to 7.4. After washing, the leucocytes were resuspended in a little of the same medium. One-tenth-millilitre fractions of each leucocyte preparation was then mixed with 0.9 ml of 0.05% trypan blue intravital stain and examined in the improved Neubauer hemacytometer (Bright line Hemacytometer, American optical Company, Scientific instrument Division, Buffalo, N.Y. 14215) for viability and cell enumeration. The viable cells did not take the stain while the dead ones were stained. The cell concentration was then adjusted to $1.5-2 \times 10^7$ cells/ml. (Falk and Zabriskie, 1971; Belsheim, 1981).

The cell suspension was mixed thoroughly and 4 non-heparinised capillary tubes of length 75 mm and internal diameter 1.0-1.2 mm were filled with the suspension. The filled capillary tubes were heat-sealed at the end having no cells, centrifuged at 250 x g for 5 minutes at room temperature using the microhaematocrit centrifuge (Hawksley, England) and cut carefully, at the cell-fluid interface, using a glass-cutter. The portions containing the cells were mounted in Sykes-Moore Chambers (Sykes and Moore, 1959). This was done in duplicates using sterile petroleum jelly. For each system, there was a control - and a test-chamber.

The control-chamber had the medium only and did not contain any antigen, while the test chamber contained the medium and the antigen.

The assembled chambers were incubated at 37°C overnight on a flat surface. The cell-migration was viewed under low-power microscope and the images of migration zones enlarged and cast onto Whattmann filter paper (18.5 mm) using the Focomat IIC enlarger (Ernst Leitz, GMBH, Wetzlar, Type 42-614-010, made in Germany). Tracings of the enlarged migration zones were then cut and weighed. The weighing was done using the Sartorius weighing machine (Sartorius-Werke AG, Feinwaage, Type 2662, made in Gottingen, Germany). The average weight for each set of papers was calculated. The percent inhibition (% MI) was then calculated using the formula of Timms (1974):

$$\% \text{ MI} = \left(1 - \frac{\text{wt. of paper from chamber with antigen}}{\text{wt. of paper from chamber without antigen}} \right) \times 100$$

since the area of migration is directly proportional to the weight of the paper traced.

3.7 VIABLE COUNTING

The bacterial suspensions were prepared as explained earlier, harvested but not killed. The viable counting was done using the method of Miles and Misra (1938). Tenfold dilutions were made ranging from 10^{-2} to 10^{-10}. For each dilution, drops were made onto petri dishes, containing MacConkey agar, using a calibrated dropper dropping 40 drops per millilitre (i.e. each drop = 25 Microlitres). Two separate drops were dispensed for each dilution. The drops were allowed to dry and the plates incubated up-side-down aerobically at 37°C overnight.

The plates were examined and counts of colony-forming-units made at the dilution giving upto 10 separate colonies. The concentration of the original harvest was calculated using the formula:

$$n \times 40 \times 10^{x} \text{ org./ml}$$

where n is the average number of colony-forming-units per drop and 10^{x} is the dilution factor.

3.8 BACTERIAL LIVER COUNTS

Liver counts were done following the method of Mackaness *et.al.* (1966). Pieces of livers were weighed (using the Harvard Trip Balance, Manufactured by Ohaus Scale Corp. Union, N. J., U.S.A.), ground and homogenised with enough sterile saline to make a 20% suspension.

This was done by adding saline to the ratio of 1.-gram liver tissue: 4 ccs. saline. The dilution factor was thus 5, and this was taken into consideration when calculating the number of organisms per gramme of tissue. Counts were done as given under "viable counting".

3.9 THE STUDY OF THE DISEASE IN GENERAL

3.9.1 Calculation of LD_{50} Values of the various S. gallinarum isolates as a measure of their Virulence

To check for the virulence of the various isolates, 20 of the characterised *S. gallinarum* isolates were randomly selected and 50% lethal dose (LD_{50}) values determined in day-old chicks for each of them. The LD_{50} experiments and calculations were carried out according to the method of Reed and Muench (1938). Briefly, pure cultures of the organisms were grown in french square bottles and viable counting of the harvest done. The original harvest was then diluted ten-fold as follows:- 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}. Nine groups consisting of five day-old-chicks each were respectively inoculated with the different bacterial concentrations. Each bird was inoculated intraperitoneally with 0.2 ml of the given suspension. The parameter monitored here was death, and the data was collected for 7 days. A tenth group, consisting of 5 uninoculated chicks was used as a control group. The different groups were colour-identified.

For each set of experiments, 2 control chicks were sacrificed before and after the start of the experiment to check their sera for any *Salmonella* agglutination and also for *Salmonella* isolation from their livers. Since all their livers were sterile and their sera showed no agglutination, it was assumed that this batch of birds was free from fowl typhoid.

3.9.2 Determination of dose-dependent terminal effects at death for day-old chicks infected with various isolates of S. gallinarum

Day-old chicks were inoculated with varying predetermined concentrations of 20 different isolates of *S. gallinarum* and the mortality recorded for each respective isolate in the following 7 days. Nine different concentrations of each isolate were inoculated into different groups of day-old chicks consisting of 5 birds each; a separate group of chicks was kept as a control for each isolate.

After death, the birds were opened aseptically and the post-mortem lesions noted. Bacterial isolation and counts were carried out for each liver in respective groups, in order to reisolate *S. gallinarum*. Birds surviving after 7 days were killed, soaked in 4% lysol and then buried.

3.9.3 Determination of the pathogenicity of S. gallinarum in adult birds

26-week-old female adults were divided into 3 groups of 20 birds each. One group was subdivided into 4 subgroups of 5 birds each and these were inoculated intraperitoneally with virulent pooled *S. gallinarum* isolates namely:- L32, L41 and L46, mixed in equal proportions, at dosages of 50 organisms, 100 organisms, 1,000 organisms and 10,000 organisms, respectively, per bird. The second group was also subdivided into 4 subgroups of 5 birds each and these were inoculated orally with the virulent pooled *S. gallinarum* isolates at dosages of 1,000 organisms, 10,000 organisms, 100,000 organisms and 1,000,000 organisms, respectively, per bird. The third group served as the control. The birds were kept in separate cages for 3 weeks and were observed for deaths, signs of sickness and subsequent re-isolation of the organisms from the liver and intestinal contents. Those birds that died were opened aseptically, post-mortem lesions noted and bacteriological examination performed on the relevant organs. Those that survived up to the end of the experiment were sacrificed by cervical dislocation, opened up aseptically and post-mortem examination and bacteriological isolation carried out.

Within the three experimental weeks, the bird's egg-production was monitored and the eggs cultured to check for presence of *S. gallinarum* organisms.

3.9.4 Pathogenesis of S. gallinarum in 45-day-old birds

45-day-old non-vaccinated birds were divided into 3 groups of 20 birds each. Birds in one group were inoculated intraperitoneally (IP) with *S. gallinarum* isolate L41 at the dosage of 9.6×10^7 organisms per bird. Those in the second group were inoculated orally with the same organism at the dosage of 3.2×10^9 organisms per bird. Those in the third group served as controls.

To trace the sequential movement of the organisms for each route, 2 birds from each group, including the controls, were sacrificed by cervical dislocation, aseptically opened and their organs processed to see whether the inoculated *S. gallinarum* organisms could be re-isolated from them. The organs investigated in the orally-challenged birds were:- liver, spleen, heartblood, crop, gizzard, duodenum and caecum. Those investigated in I.P.-challenged birds were:- liver, spleen, heartblood and caecum; while those investigated in control birds were:- liver and spleen. The sacrifices were done after intervals of 3 hours, 6 hours, 1 day, 2 days, 3 days, 6 days, 7 days, 8 days, 9 days and 10 days post-inoculation.

The respective organs of the two birds from one group, sacrificed together, were processed separately and the average count taken. The heart-blood was dropped directly onto MacConkey medium using a calibrated dropper delivering 25 µl. The drop was allowed to dry and the plate incubated aerobically, up-side-down, at 37°C overnight. The crops and gizzards were opened and contents emptied into sterile petri-dishes, respectively. The contents of the duodenal loop and the caecum were squeezed out, using forceps, and put in sterile petri-dishes, respectively. The contents were weighed and made up to 20% suspension using sterile saline. Doubling dilutions were prepared starting at 1/5. The viable counting was then done as described earlier. The livers and spleens were processed as described earlier.

3.10 THE STUDY OF THE IMMUNE RESPONSE TO SALMONELLA GALLINARUM ISOLATES

3.10.1 Development of new vaccine strain

From the LD_{50} experiments, *S. gallinarum* isolate L46, with an LD_{50} of one (1) organism, was chosen as the experimental vaccine strain. Preliminary studies showed that at a dose of 1,000 organisms per adult bird intramuscularly, L46 behaved as if it was non-pathogenic to the birds. This dosage was used henceforth to study the immune response to L46 as a model for *S. gallinarum* infections.

The currently used *S. gallinarum* vaccine strain is CN 180.

3.10.2 Immune response of adult birds to CN 180 and L46 vaccines monitored over a 37-week period

Nine 15-week-old cockerels were divided into 3 groups of 3 chickens each. One group, with cockerels numbered 8, 19 and 21, were vaccinated with the currently used Vet. Labs. vaccine strain, CN 180, at the dosage suggested by the producers i.e. 1 ml. of broth containing 1,000 organisms per millilitre. The other group, with cockerels numbered 23, 10 and 20 were vaccinated with a virulent isolate of *S. gallinarum* L46, at a non-infective dose of 1,000 organisms intramuscularly. The last group, with cockerels numbered 12, 15 and 17, served as controls. The groups of birds were kept in separate cages. Before choosing the 9 cockerels, 5 birds from the flock were screened with MIT and were found to be negative.

Bleeding both for sera (for IHA) and for cells (for MIT) was done every week up to 7th week post-vaccination, then, after every alternate week 3 times, and thereafter once a month. At the same time, the birds were screened with the RWBPT. IHA was done using fresh sheep red-blood-cells.

3.11 A STUDY OF THE VARIOUS WAYS OF CONTROLLING THE DISEASE

3.11.1 Effectiveness of Vaccination

3.11.1.1 Protective ability of CN 180 - Vaccine to day old chicks challenged with virulent S. gallinarum

This experiment was set up to determine the ability of the currently-used vaccine strain, CN 180, to confer protection against fowl typhoid, to day-old chicks. Thirty (30) day-old chicks were divided into 6 groups of 5 birds each. Five (5) groups were vaccinated intraperitoneally with 0.2 ml. of varying concentrations of CN 180 organisms, ranging from 2.14×10^3 to 2.14×10^7 org./ml., while the 6th group served as controls. After two weeks, the birds were challenged with a virulent strain of *S. gallinarum*, isolate L46. This was given at a concentration of 2.53×10^8 org/ml.

Three birds from each of the 6 groups were inoculated intraperitoneally with 0.2 ml. of the suspension of isolate L46 prepared as described above i.e. each bird was exposed to 5.06×10^7 organisms. The remaining birds in each group served as controls. The true controls, i.e. not vaccinated and not challenged, were designated "Control A", while the vaccinated controls i.e. vaccinated but not challenged, were designated "Control B". The different groups of birds were housed separately and the pathogenicity monitored by death and isolation of the organism. The experiment ran for 38 days.

3.11.1.2 Comparative Efficacy of CN 180 - and L46 - Vaccines in adult birds challenged with virulent S. gallinarum

Two groups of 15-week-old pullets each consisting of 32 birds were vaccinated as follows: one group was vaccinated with the currently-used Vet. Labs. vaccine strain, CN 180, at the dosage suggested by the producers i.e. 1 ml. of a broth suspension containing 1,000 org./ml. The other group was vaccinated with a virulent isolate of *S. gallinarum*, L46, at a non-infective dose, as calculated earlier. Here, 1,000 organisms were injected intra-muscularly per bird. A control group consisted of 23 pullets.

Eight (8) birds from each vaccinated group were challenged with *S. gallinarum* isolate L46, intraperitoneally, after periods of 4 weeks, 8 weeks, 13 weeks and 17 weeks, post-vaccination. The challenge dose ranged from 1,400 to 2,000 organisms per bird. After challenge, 2 birds from each challenged group were sacrificed on days 1, 2, 3 and 6 post-challenge, for the first and second challenges; days 1, 2, 5 and 6, post-challenge, for the third challenge, and days 4, 6, 10 and 12, post-challenge, for the fourth challenge. The birds were weighed using the Avery weighing machine No. 1301 BCD (made in England by W. and T. Avery Ltd., Birmingham) and then their livers and spleens were removed aseptically. These were also weighed using Harvard Trip Balance (manufactured by Ohaus Scale Corp. Union, N. J., U.S.A.) and the liver/spleen hypertrophy indices were calculated as given by Waiyaki (1974).

The bacterial counts of the livers from the experimental birds and also from the control birds were done for each set of birds sacrificed. Since there were 4 sacrifices for each challenge, the count per sacrifice was to give an indication of the liver clearance (reduction) capacity with respect to *S. gallinarum* organisms. As the birds were killed, for the purposes given above, blood was collected for serum and for cells to check for the status of both humoral and cell-mediated immunity at that particular stage post-vaccination. RWBPT was also done. The sera were screened using IHA and the cells, mainly lymphocytes and macrophages, were used to run MIT. MIT was done mainly in the first and second challenges and occassionally in the third and fourth challenges. IHA was done using fresh sheep red-blood-cells and *S. gallinarum* as the antigen.

3.11.2 Antibiotic sensitivity testing of the S. gallinarum isolates

S. gallinarum isolates were tested for antibiotic sensitivity using tetracycline, erythromycin, chloramphenicol, furazolidone, neomycin, penicillin, cloxacillin, ampicillin, streptomycin, sulphur compounds, co-trimoxazole, nitrofurantoin, nalidixic acid and gentamycin (Oxoid (UK) Ltd., Basingstoke, England). The method used was the diffusion technique (WHO Report, 1979) using antibiotic impregnated discs (multodiscs).

To ensure uniform bacterial seeding, for the test, organisms were first grown in pure culture on MacConkey agar. A piece of agar containing 3 colonies of an 18-hour culture was then cut out using a sterile scapel blade and this was added to 10 millilitres of sterile distilled water. The organisms were dislodged from the agar by vigorous shaking, making a suspension of about 10^6 organisms per millilitre. Fifty microlitres (µl) of the suspension was then poured onto the sensitivity test agar No. 1 (Gibco, Europe) and sterile cotton swab was used to spread the suspension all over the surface of the medium, finally encircling around the edge of the agar surface. The inoculum was left to dry for a few minutes at room temperature with the lid closed. The antibiotic discs were then placed on inoculated plates using a pair of sterile forceps. The plates were incubated at 37°C overnight and the diameter of each zone measured and recorded in millimetres. The results were interpreted according to the critical parameters set by McGhie and Finch (1975), as follows: inhibition zones up to 2 millimetres from the edge of the disc to the inhibition zone front (13mm diameter) were taken as resistant, and those beyond 2 millimetres (13mm diameter) were taken as sensitive.

Antibiotic sensitivity testing was also carried out on standard cultures, namely: Oxford *Staphylococcus aureus* (NCTC No. 6571) as control for gram-positive organisms and *Escherichia coli* type H 10407 (serotype 0:78 H:11) as control for gram-negative organisms. These were obtained from the Kenya Medical Research Institute, Nairobi. The antibiotic discs were stored at - 20°C as suggested by Parry (1977).

3.11.3 Disinfectant Sensitivity Testing of The Various S. gallinarum isolates

A diffusion technique using wells punched into the sensitivity test agar No. 1 (Gibco, Europe) was used to monitor the inhibitory effects of various dilutions of 6 commonly used disinfectants namely Lysol^R (Alpha Chemicals Ltd., Lunga Road, Nairobi - a cresol and soap solution), Pynol^R (Wellcome Kenya Ltd., Kabete - a chlorinated phenol), Kerol^R (Wellcome Kenya Ltd., Kabete - containing not less than 38% v/v of neutral tar acids), Biodan^R (Wellcome Kenya Ltd., Kabete - an iodophor), Municipal fluid^R (Wellcome Kenya Ltd, Kabete - containing not less than 38% tar acids), and Bromosept^R (TDA).

The seeding was done in the same way as for the antibiotic sensitivity testing. Fifty microlitres of the *S. gallinarum* bacterial suspension at a concentration of about 10^6 organisms per ml. were spread with a sterile cotton swab over the agar. After seeding, wells were punched using a sterile 6mm diameter well-cutter. Various dilutions of the disinfectants were dispensed into different wells, using one petri-dish per disinfectant and running them in duplicates. Each well was loaded with 50 µl of the relevant disinfectant dilution. The plates were incubated upright at 37°C overnight and the diameters of each zone measured and recorded in millimetres. The results were interpreted in the same way as the antibiotic sensitivity, that is:- inhibition zones up to 2 millimetres from the edge of the well to the inhibition zone front (10 mm. diameter) were taken as resistant, and beyond 2 millimetres (10 mm. diameter) were taken as sensitive. Disinfectant sensitivity testing was also carried out on standard cultures, namely: Oxford *Staphylococcus aureus* (NCTC No. 6571) as control for gram-positive bacteria and *Escherichia coli* type H 10407 (serotype 0:78 H:11) as control for gram-negative bacteria. These were obtained from the Kenya Medical Research Institute, Nairobi.

3.12 OTHER INVESTIGATIONS

3.12.1 Phage typing of S. gallinarum isolates

3.12.1.1 Phages Used

Five phages labelled 1, 2, 3, 4 and 5 were used to carry out the typing exercises. The phages were supplied by Dr. R. Rowe of the Reference Laboratories of International Federation of Enteric Phage Typing, Central Public Health Laboratory, 175 Colindale Avenue, London NW 9 5 HT, United Kingdom.

3.12.1.2 Host Bacteria

The host bacteria for the phages were as follows: *S. gallinarum* isolate L64 for phages 1 and 2, isolate L59 for phage 3, and isolate L29 for phages 4 and 5. These were used at concentrations of 7.0×10^9, 8.4×10^9 and 1.28×10^{10} org./ml., respectively. These were grown in peptone broth and MacConkey agar.

3.12.1.3 Phage Propagation and Harvesting

This was done following the method given by Corbel and Thomas (1980), using the respective host bacteria and media.

3.12.1.4 Phage Titration

The titration was done according to Rovozzo and Burke (1973), using both hard and soft agar. The phages were used at concentrations of 4.0×10^5, 1.1×10^8, 7.2×10^6, 4.0×10^5 and 2.5×10^6 plaque-forming units per millilitre, respectively.

3.12.1.5 Phage Typing

The phage-typing was done according to Corbel and Thomas (1980). Briefly, thick streaks of each isolate to be typed were made on blood agar and the phage suspension dropped on the streak, using a standard dropper delivering 25 µl. The drops were left to dry and the plates incubated, up-side-down, at 37°C overnight. The reading was done based on clearing of the lawn. The reactions were scored on an arbitrary scale of 1+ to 4+, whereby complete clearing was assigned the value of 4+ and faint clearing a value of 1+.

3.12.2 A STUDY OF THE IMMUNE REACTION TO THE DIFFERENT SOMATIC ANTIGENS OF S. GALLINARUM

3.12.2.1 A Comparative Study of the Various S. gallinarum somatic antigens in relation to the antibody titres they elicit.

S. gallinarum has 3 somatic antigens, namely:- 1, 9 and 12. It was found necessary to determine presence or otherwise of differences in the immune response to these antigens. This may give an indication of the possible protective antigen in fowl typhoid. Antigens containing only one of the *S. gallinarum* antigens were prepared. The following *Salmonella* serotypes were chosen to represent the individual antigens:- *S. strasbourg*, which has an antigenic formula of 9,46:d:1,7, (ii) *S. senftenberg*, which has an antigenic formula of

1, 3, 19: g, s, t; and (iii) *S. Kiambu*, which has an antigenic formula of 4, 12: Z: 1, 5. These represented antigens 9, 1 and 12, respectively. The *S. strasbourg*, strain 627, was obtained from the National Institute of Public Health. Oslo, Norway, while *S. senftenberg* and *S. Kiambu* were obtained from the Veterinary Research Laboratories, Kabete. A pooled antigen, consisting of all the 3 antigens, i.e. 1, 9 and 12, was prepared using *S. gallinarum* isolate L25. The antigens were tested against reference sera for *Salmonella* polyvalent 'o', *Salmonella* factors 1, 9 and 12, using IHA, to show that there were no cross-reactions between them.

Sera from naturally infected birds as well as field-vaccinated birds were screened with IHA using the different antigens prepared above. The sheep red-blood-cells (RBC's) were collected and formalin-fixed as described earlier. Four (4) batches of RBC's were coated with the 4 antigens, respectively, and these were used to run IHA using the procedure described earlier.

3.12.2.2. Humoral and Cellular immunity to the different S. gallinarum somatic antigens as assayed in experimental cockerels

Nine (9)-month-old cockerels were vaccinated intramuscularly with the currently-used veterinary laboratories' vaccine strain, CN 180, and the immune response monitored by various serological tests, namely: RWBPT, SAT, TAT and IHA, and also by MIT. The 4 antigens mentioned in section 3.12.2.1 were used in SAT. TAT and MIT; the commercially obtained stained antigen was used for RWBPT; and the pooled antigen, i.e. *S. gallinarum* strain L25, was used for IHA. Thus, for SAT, TAT and MIT, each serum or cell-batch was screened using the 4 antigens separately. The MIT was monitored from day 26 post-vaccination. IHA was done using fresh sheep red-blood-cells.

The bleedings were carried out from the wing-vein on days 3, 6, 10, 13, 19, 26, 32, 39 and 46 post-vaccination. About 25 birds were vaccinated and 25 kept as controls. At the time of bleeding, any 3 of the vaccinated birds and 3 of the control birds, picked at random, were bled for sera and cells. Sera and cells from the 3 birds were pooled, respectively, and tested. Care was taken not to bleed the same birds repeatedly. This ensured that a new set of birds was bled every time. Three birds confirmed to be having fowl typhoid were bled and their sera and cells pooled and run like the others.

* 3.13 STATISTICAL ANALYSIS

The test statistics used here were the McNemar's test for correlated proportions and the general chi-square test (Remington and Schork, 1970).

4. RESULTS

4.1 MEDIUM LETHAL DOSE

The results for the LD_{50} determinations are shown in Table 1. There are differences in virulence between the various isolates assessed. Of the 20 isolates tested for virulence, 8(40%) gave an LD_{50} of one (1), 3(15%) gave an LD_{50} of two (2), 2(10%) gave an LD_{50} of three (3) and the rest (35%) gave LD_{50}'s of 6 and above. One isolate, L23, gave an LD_{50} of 2.6×10^4 organisms. The currently used fowl-typhoid vaccine, strain CN 180, was found to be non-pathogenic to day-old chicks.

4.2 DETERMINATION OF DOSE - DEPENDENT TERMINAL EFFECTS AT DEATH FOR DAY-OLD CHICKS INFECTED WITH VARIOUS ISOLATES OF S. GALLINARUM.

Towards the establishment of the effect of various doses of *S. gallinarum* isolates on the terminal effects at death for day-old chicks, Table 2 shows the average death-pattern shown by day-old chicks infected with various concentrations of *S. gallinarum* isolates and Figure 1 illustrates the average death-rate per concentration. The detailed results are given in Appendix 1.

Table 1: LD_{50} VALUES OF 20 S. GALLINARUM ISOLATES MONITORED IN DAY-OLD CHICKS

Isolate	Number actually injected i.e. contained in the 0.2ml of the suspension $\equiv LD_{50}$ value	Overall concentration of the suspension
L21	21	101 org/ml.
L56	3	12 org/ml.
L32	1	5 org/ml.
L31	1	4 org/ml.
L41	1	1 org/ml.
L55	17	85 org/ml.
L7	328	1.6×10^3 org/ml.
L26	800	4×10^3 org/ml.
L23	2.6×10^4	1.3×10^5 org/ml.
L47	3	11 org/ml.
L46	1	3 org/ml.
L3	1	4 org/ml.
L33	1	3 org/ml.
L71	2	8 org/ml.
L25	2	8 org/ml.
L72	1	2 org/ml.
L42	9	45 org/ml.
L65	2	6 org/ml.
L37	6	27 org/ml.
L24	1	3 org/ml.

Key org/ml means organisms per millilitre

Table 2: AVERAGE DAILY MORTALITY OF DAY-OLD CHICKS INFECTED WITH S. GALLINARUM ISOLATES AT VARYING CONCENTRATIONS.

Days	Number of dead birds							
	10^{8*}	10^{7*}	10^{6*}	10^{5*}	10^{4*}	10^{3*}	10^{2*}	10^{1*}
1	0	0	0	0	0	0	0	0
3	5	4	2	2	1	1	0	0
5	-	1	3	2	3	2	2	2
7	-	-	-	1	1	2	2	1

Key * Dose concentration in org/ml.

0 means "no deaths in infected birds"

- means "all the birds inoculated died"

AVERAGE DAILY MORTALITY OF DAY OLD CHICKS INFECTED WITH S. GALLINARUM ISOLATES AT VARYING CONCENTRATIONS.

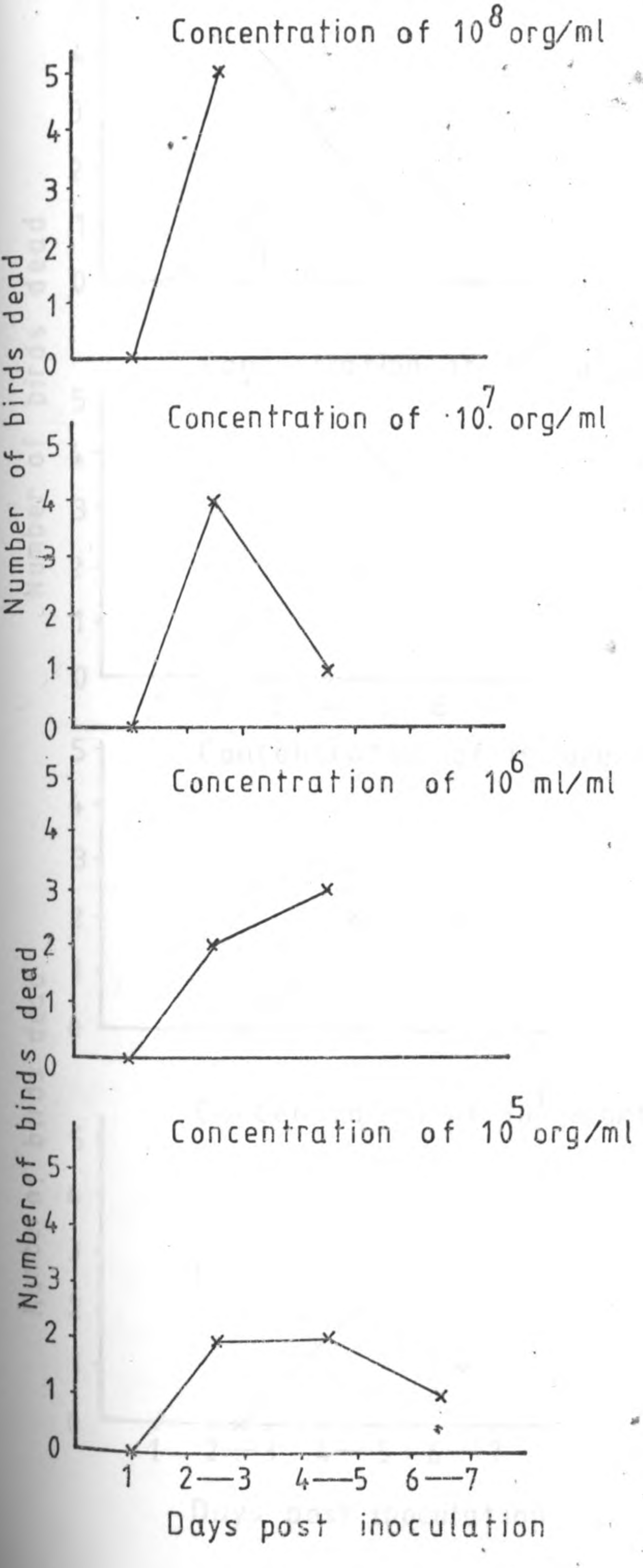
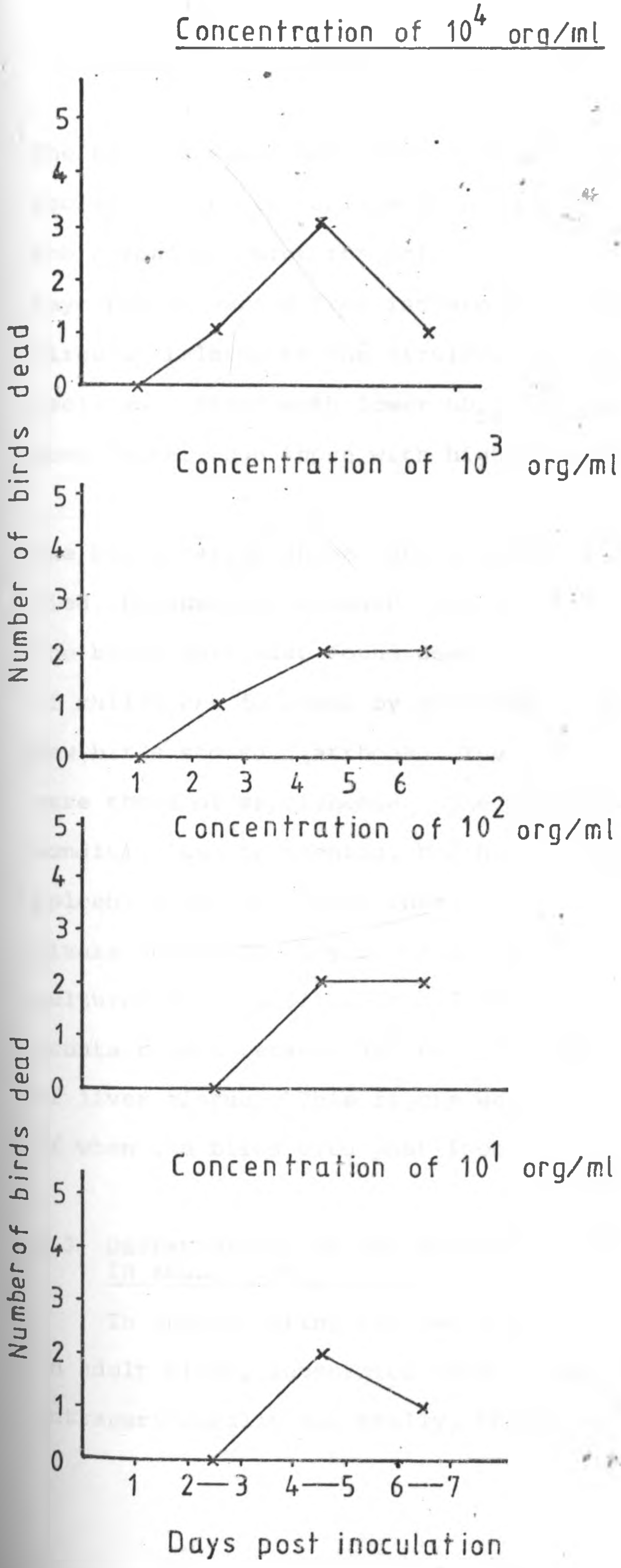


FIGURE 1


Concentration of 10^4 org/ml
Concentration of 10^3 org/ml
Concentration of 10^2 org/ml
Concentration of 10^1 org/ml
Number of birds dead
Number of birds dead
0
1
2
3
4
5
1
2
3
4
5
6
1
2—3
4—5
6—7
Days post inoculation

The results show that, higher doses of the infecting isolate killed the chicks at a faster rate than lower doses. The spread at which the chicks were killed over the 7 days period varied from isolate to isolate and was directly related to the virulence of the respective isolates. Those with lower LD_{50} values killed the birds much faster than those with higher values.

The birds rarely showed any clinical signs before they died, because the disease took an acute course. Most of the birds were just found dead, while a few showed signs of chilliness followed by prostration and death. Very few birds showed diarrhoea. The post-mortem lesions were those of septicaemia. The chicks were in fair condition and on opening, had haemorrhages in the liver, spleen, lungs and intestines. Some birds, had congested livers only. The livers of the dead birds yielded pure cultures of *S. gallinarum* and the average liver bacterial counts ranged between 10^7 to 10^8 organisms per gramme of liver tissue. This figure was consistent regardless of when the birds died post-inoculation.

4.3 DETERMINATION OF THE PATHOGENICITY OF S. GALLINARUM IN ADULT BIRDS

In investigating the pathogenicity of *S. gallinarum* in adult birds, inoculated through two routes, namely: intraperitoneally and orally, the mortality rate for the intraperitoneally - inoculated birds was as given in Table 3. The lesions found in the dead birds were typical of fowl typhoid, that is: microabscesses in the liver, or liver with greenish metallic sheen, enlarged spleen, ovaries affected to varying degrees:- some had well-developed ova, others had atrophied ova. The ova looked congested. Some birds showed catarrhal enteritis. The livers yielded pure cultures of *S. gallinarum* at counts in the region of 10^8 to 10^9 organisms per gramme of liver tissue.

Birds that survived to the end of the experiment showed different lesions, on opening. The remaining bird in the group that had been injected with 1,000 organisms looked obviously sick and had whitish diarrhoea. On opening, it had congested liver and atrophied ova. On bacteriological examination, this bird's liver yielded *S. gallinarum* at counts of 1.14×10^5 organisms per gramme of tissue. One bird from the group that had received 100 organisms looked normal but, on opening, had greenish enlarged liver, enlarged spleen and "fluidy" ovaries. The liver in this bird yielded *S. gallinarum* at 1.2×10^4 organisms per gramme of tissue. The rest of the birds looked normal, and, on opening, the majority had well developed, although congested, ova; some even had fully-developed eggs in the oviduct, while the remaining few had atrophied ova. A few of these birds showed congested livers while the majority had livers which looked normal. Livers from these birds did not yield any bacteria.

Table 3: THE MORTALITY RATE OF BIRDS THAT WERE CHALLENGED INTRAPERITONEALLY WITH S. GALLINARUM AND MONITORED FOR 3 WEEKS

Challenging dose	No.dead	No.alive	% dead
50 organisms	0	5	0
100 organisms	0	5	0
1,000 organisms	4	1	80
10,000 organisms	5	0	100

Orally - inoculated birds and controls did not show any sign of sickness up to the day they were sacrificed. Their organs looked normal and the faeces did not yield any *Salmonella*, on culture.

The number of eggs collected during the experimental period for each group was as given in Table 4. There was a drop in egg-production in the group that was inoculated intraperitoneally (I.P.) and not in that inoculated orally. The egg-production of the orally-inoculated birds was more-or-less the same as that of the control birds. The cultured eggs did not yield *S. gallinarum*.

4.4 PATHOGENESIS OF S. GALLINARUM IN 45-DAY-OLD BIRDS

The pathogenesis of *S. gallinarum* as shown by isolation of the organism from various organs in I.P.- inoculated and orally-inoculated birds is given in Tables 5 and 6, respectively.

The I.P.-inoculated birds had the *S. gallinarum* organisms in their livers and spleens 3 hours post-inoculation. The bacterial counts fluctuated from 10^4 organisms per gramme of tissue (org/g) in the liver and 10^5 org/g in the spleen at 3 hours post-inoculation to 10^2 org/g and 10^3 org/g in the liver and spleen, respectively, at 7 days post-inoculation. The highest liver/spleen counts were at 3 hours post-inoculation where there were $3.28\text{x}10^4$

Table 4: COMPARISON OF CUMULATIVE EGG-PRODUCTION IN THE THREE GROUPS OF BIRDS OVER THE EXPERIMENTAL PERIOD OF 3 WEEKS

1. P. Challenged birds	Orally-challenged birds	Controls
21 eggs	51 eggs	52 eggs

Key: 1. P. means "intraperitoneal"

Table 5: ISOLATION OF S. GALLINARUM FROM VARIOUS ORGANS AFTER I.P. INOCULATION OF 45-DAY-OLD NON-VACCINATED BIRDS WITH ISOLATE L41

Time post-inoculation	Heart-blood	Liver	Spleen	Caecum
3 hours	-	3.28×10^4	1.32×10^5	-
6 hours	1.8×10^2	2.2×10^4	6.4×10^3	-
24 hours	-	5.6×10^3	1.84×10^4	-
2 days	-	8.0×10^2	3.0×10^2	-
3 days	-	1.6×10^3	4.8×10^3	-
6 days	-	-	-	-
7 days	-	4×10^2	1.2×10^3	-
8 days	-	-	-	-
9 days	-	-	-	-
10 days	-	-	-	-

Key: - means "no organism isolated"

NB Heart blood - counts are given as the number of organisms per millilitre of blood

Liver and spleen - counts are given as the number of organisms per gramme of tissue.

Table 6: ISOLATION OF S. GALLINARUM FROM VARIOUS ORGANS AFTER ORAL INOCULATION OF 45-DAY-OLD NON-VACCINATED BIRDS WITH ISOLATE L41

Time post-inoculation	Crop	Gizzard	Duodenum	Caecum	Liver	Spleen	Heart blood
3 hours	-	-	-	-	-	-	-
6 hours	1.44×10^4	-	-	-	-	-	-
24 hours	-	-	-	-	8×10^2	2×10^2	-
2 days	-	-	-	-	-	-	-
3 days	-	-	-	-	-	-	-
6 days	-	-	-	-	-	-	-
7 days	-	-	-	-	-	-	-
8 days	-	-	-	-	-	-	-
9 days	-	-	-	-	-	-	-
10 days	-	-	-	-	-	-	-

Key - means "no organism isolated"

NB The counts are given as the number of organisms per gramme of tissue.

and 1.32x10^5 org/g, respectively. From day 8 Post-inoculation onwards, no *S. gallinarum* organisms were detected from the livers and spleens. One notes that no organisms were re-isolated from the livers and spleens of the two birds that were sacrificed on day 6 post-inoculation. Organisms to the count of 1.8 x 10^2 org/ml. were detected in the heartblood at 6 hours post-inoculation only. There were no *S. gallinarum* organisms re-isolated from the caecal samples processed.

The orally-inoculated birds had the organisms detected in the crop at a concentration of 1.44x10^4 org/g at 6 hours post-inoculation only. The organisms were re-isolated from the livers and spleens at concentrations of 8.0x10^2 and 2.0x10^2 org/g, respectively, 24 hours post-inoculation. After this, there were no other re-isolations made from the samples collected. The gizzard, duodenum, caecum and heart-blood samples did not yield any *S. gallinarum*. One notes, here, that while the organisms had localised in the liver/spleen by 3 hours post-inoculation in the I.P. - inoculated birds, they did so 24 hours post-inoculation in orally-inoculated birds.

The control birds did not yield any *S. gallinarum*.

4.5 IMMUNE RESPONSE OF ADULT BIRDS TO CN 180 AND L46 VACCINES MONITORED OVER A 37-WEEK PERIOD

Tables 7, 8 and 9 give the MIT, IHA and RWBPT results for the vaccinated cockerels, and figures 2 and 3 give the graphical representations of the MIT and IHA results. A detailed table is given in Appendix 2. There was immune response towards the two vaccines, the response increasing to a maximum of 65% migration inhibition in CN 180 - vaccinated birds and 76% in L46 - vaccinated birds 9 weeks post-vaccination, after which there was a sharp fall from a migration inhibition of 65%/67% on week 13, post-vaccination, in CN 180 - and L46 - vaccinated birds, respectively, to 27% on week 17 post-vaccination. The response, as measured by percent macrophage migration inhibition, then fluctuated as time went on. L46 seemed to be more effective than CN 180 in maintaining the response. It is interesting to note that the control birds also showed some MIT responses of upto 27%.

Overall, the L46 - vaccinated birds showed higher titres than the CN 180 - vaccinated birds, the peak titre of 44,117 being demonstrated on week 17 post-vaccination. The titres fluctuated a lot throughout the experimental period. The CN 180 - vaccinated birds showed a more gradual increase in titre and there were less fluctuations than in L46 - vaccinated birds. The control birds also gave titres up to 32 to *S. gallinarum*.

Table 7: MIT RESPONSE FOR COCKERELS VACCINATED WITH S. GALLINARUM STRAINS CN 180 AND L46, GIVEN AS % MIGRATION

Bird Number	Percent Migration Inhibition									
	Wk.1	2	4	5	7	9	13	17	21	37
Birds Vaccinated with CN 180										
8	N1	-	-	61.01	-	-	66.92	8.02	-	N1
19	7.61	29.66	3.93	47.22	-	65.08	46.53	45.5	51.71	-
21	19.05	31.61	14.1	67.55	-	64.37	82.77	-	35.05	14.3
Birds Vaccinated with L46										
23	5.74	-	-	72.87	-	73.66	95.73	19.89	43.28	29.35
10	26.52	16.78	-	72.42	-	79.16	44.69	61.35	-	80.76
20	37.81	34.18	-	67.72	-	-	61.09	NI	44.33	-

Key

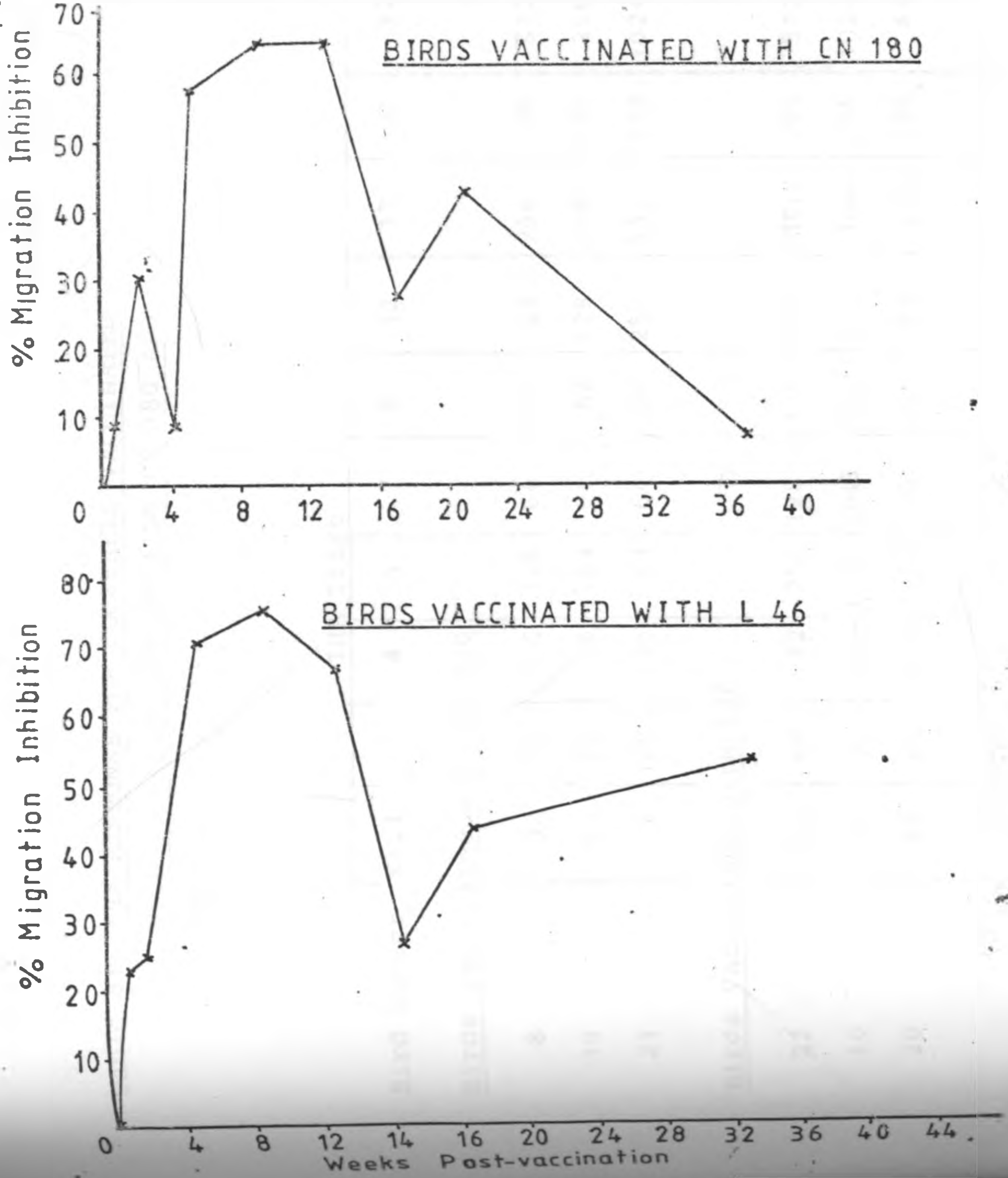
- means "not done" or "set up spoilt"

NI means "no inhibition

W wk means "week"

FIGURE 2: AVERAGE % MIGRATION INHIBITION PER GROUP OF COCKERELS

FIGURE 2



BIRDS VACCINATED WITH CN 180
% Migration Inhibition
70
60
50
40
30
20
10
0
4
8
12
16
20
24
28
32
36
40
BIRDS VACCINATED WITH L 46
% Migration Inhibition
80
70
60
50
40
30
20
10
0
4
8
12
14
16
20
24
28
32
36
40
44
Weeks Post-vaccination

Table 8: IHA RESPONSE FOR COCKERELS VACCINATED WITH S. GALLINARUM STRAINS CN 180 AND L46

	IHA Titre									
Bird Number	Wk.1	2	4	5	7	9	13	17	21	37
Birds Vaccinated with CN 180										
8	32	16	256	128	64	128	64	256	128	512
19	64	64	256	64	64	64	128	128	512	256
21	32	128	128	64	64	128	256	512	512	1024
Birds Vaccinated with L46										
23	128	64	32	256	128	128	512	131072	256	512
10	16	32	1024	1024	2048	1024	512	1024	256	1024
20	64	64	128	64	256	256	64	256	128	64

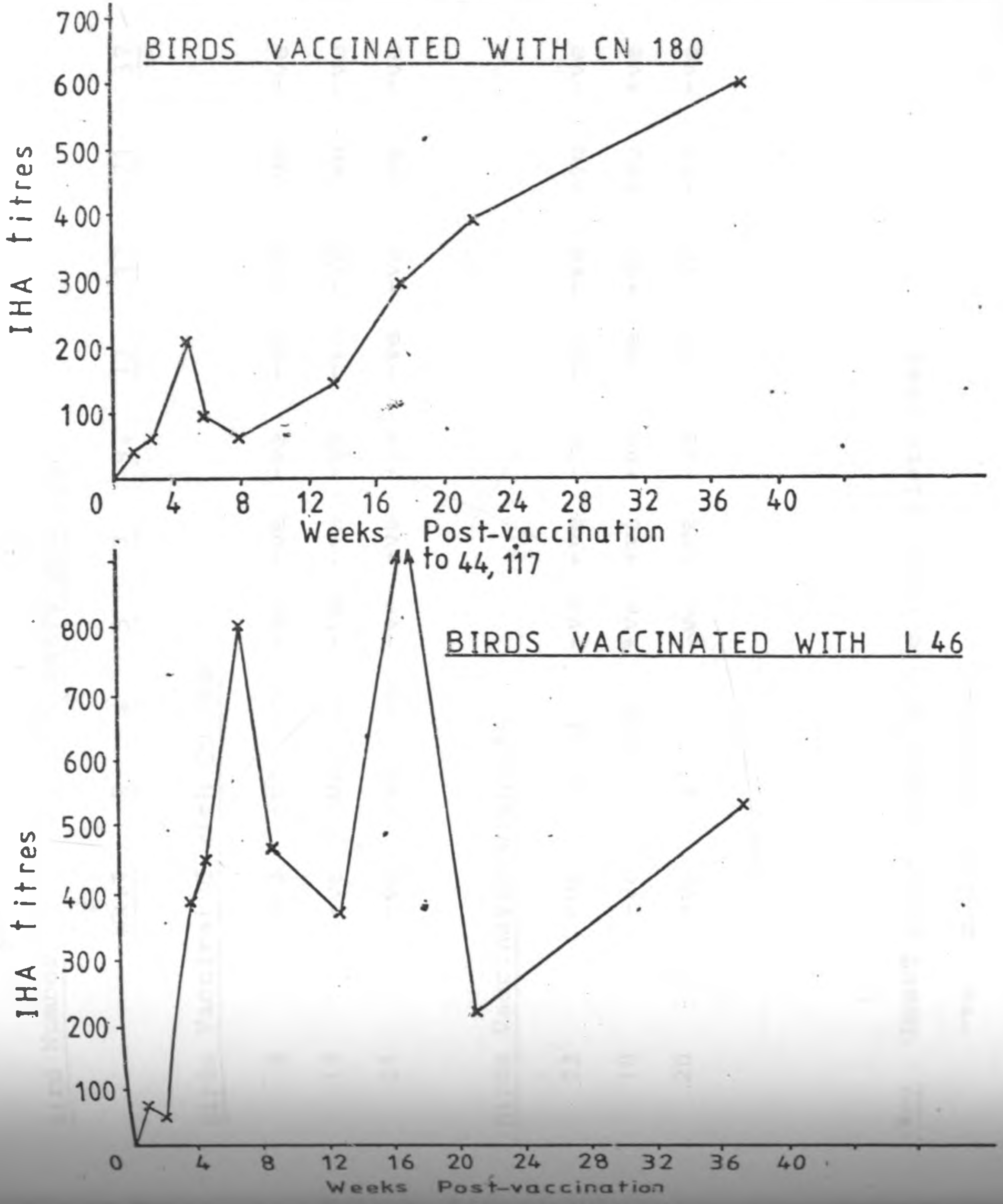
Key IHA means "indirect haemagglutination test"

wk means "week"

W

FIGURE 3: AVERAGE IHA TITRES PER GROUP OF COCKERELS

FIGURE 3



BIRDS VACCINATED WITH CN 180

BIRDS VACCINATED WITH L 46

Table 9: RWBPT RESPONSE FOR COCKERELS VACCINATED WITH S. GALLINARUM STRAINS CN 180 AND L46

Bird Number	RWBPT Results Wk. 1	2	4	5	7	9	13	17	21	37
Birds Vaccinated with CN 180										
8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
19	-ve	-ve	±	-ve	-ve	-ve	-ve	-ve	-ve	-ve
21	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Birds Vaccinated with L46										
23	-ve	-ve	±	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10	-ve	±	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
20	-ve	±	±	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Key RWBPT means "rapid whole blood plate test"
-ve means "negative"
± means "suspiciously positive"
+ve means "positive"
wk means "week"

These IHA results matched well with the MIT results, where the L46 - vaccinated birds gave in general, higher MIT values than the CN 180 - vaccinated birds. This shows that the isolate L46 induces a more intense immunological reaction than CN 180. One of the birds vaccinated with L46 (bird No.10) developed good immunity as indicated by the high MIT values and IHA titres it gave, starting from low values; and also by its strong positive agglutinations with RWBPT from week 4 onwards while all other birds gave negative results. Bird No.23, also from the L46 - vaccinated group, gave the highest IHA titre of 131,072 at week 17 post-vaccination. One notes, here, that although the IHA titre of this bird was higher than that of bird No.10, at this particular time, bird No.23 gave negative RWBPT result while bird No.10 gave positive RWBPT result.

4.6 PROTECTIVE ABILITY OF CN 180 - VACCINE TO DAY-OLD CHICKS CHALLENGED WITH VIRULENT S. GALLINARUM

Protection was monitored by survival after challenging the birds with the virulent *S. gallinarum* organisms and Table 10 gives the death-rate for the different groups of birds. There was very little protection rendered by the vaccine to the day-old chicks. The dose given did not affect the outcome considerably.

Those that died yielded liver counts of *S. gallinarum* ranging from 10^7 to 10^9 org/g. The control birds did not yield any *Salmonella*.

Table 10: MORTALITY OF CHICKS VACCINATED WITH CN 180 AT ONE-DAY-OLD AND CHALLENGED AT ONE-WEEK OF AGE WITH VIRULENT ISOLATE OF S. GALLINARUM

Dose-group	No.Challenged	No.dead	% dead
2.14×10^{7} org/ml.	3	2	66.7
2.14×10^{6} org/ml.	3	3	100.0
2.14×10^{5} org/ml.	3	1	33.3
2.14×10^{4} org/ml.	3	2	66.7
2.14×10^{3} org/ml.	3	3	100.0
Challenged controls	3	3	100.0
Control A	0	0	-
Control B	0	0	-

Key Control A were control birds that were not vaccinated and not challenged.

Control B were control birds that were vaccinated but not challenged.

4.7 COMPARATIVE EFFICACY OF CN 180 - AND L46 - VACCINES IN ADULT BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM

Liver hypertrophy indices, spleen hypertorphy indices and liver bacterial counts for the challenged controls, birds vaccinated with CN 180, and those vaccinated with L46 were as given in Tables 11, 12, 13, 14, 15, 16, 17, 18, 19, respectively. Appendix 3 gives the composite data of all the observations done. The liver:body weight and spleen: body weight ratios for the normal birds were found to be 2.3% and 0.176%, respectively.

L46 and CN 180 - vaccinated birds showed greater resistance to the colonisation of the organisms in the liver than the controls (Tables 17, 18, 19) since, in the first challenge, there was a large number of organisms in the control livers on days 1 and 3 post-challenge. These were as high as 16,000 and 9,600 organisms per gramme of liver tissue, respectively. The CN 180 - vaccinated birds had a time lag of 2 days before there was colonisation in the liver. The number of organisms per gramme of liver tissue was 650 by the 3rd day and 1,000 by the 6th day post-challenge. The L46 - vaccinated birds had a time lag of 5 days before there was colonisation in the liver. The number of organisms per gramme of liver tissue was 8,800 by the 6th day post-challenge. The lag period could be attributed to prompt phagocytosis by macrophages as the organisms gained entry into the body. The challenging dose was 1,600 organisms per bird intraperitoneally.

Table 11: AVERAGE LIVER HYPERTROPHY INDICES FOR UNVACCINATED BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM ISOLATE

Day Post-Challenge	Liver hypertrophy index
1	NH
2	NH
3	NH
6	H

Key NH means "no hypertrophy", which denotes liver hypertrophy indices of 1 and below

H means "hypertrophy", which denotes liver hypertrophy indices of more than 1

Table 12: AVERAGE LIVER HYPERTROPHY INDICES FOR CN 180-VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Liver hypertrophy index
1st challenge: 4 weeks post-vaccination	
1	NH
2	H
3	NH
6	H
2nd challenge: 8 weeks post-vaccination	
1	NH
2	H
3	NH
6	NH
3rd challenge: 13 weeks post-vaccination	
1	NH
2	NH
5	H
6	H
4th challenge: 17 weeks post-vaccination	
4	H
6	H
10	H
12	H

Key NH means "no hypertrophy", which denotes liver hypertrophy indices of 1 and below

H means "hypertrophy", which denotes liver hypertrophy indices of more than 1

Table 13: AVERAGE LIVER HYPERTROPHY INDICES FOR L46-VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Liver hypertrophy index
1st challenge: 4 weeks post-vaccination	
1	NH
2	NH
3	NH
6	H
2nd challenge: 8 weeks post-vaccination	
1	NH
2	NH
3	H
6	H
3rd challenge: 13 weeks post-vaccination	
1	NH
2	H
5	H
6	H
4th challenge: 17 weeks post-vaccination	
4	H
6	H
10	H
12	H

Key NH means "no hypertrophy", which denotes liver hypertrophy indices of 1 and below

H means "hypertrophy", which denotes liver hypertrophy indices of more than 1

Table 14: AVERAGE SPLEEN HYPERTROPHY INDICES FOR UNVACCINATED BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Spleen hypertrophy index
1	NH
2	H
3	H
6	H

Key NH means "no hypertrophy" which denotes spleen hypertrophy indices of 1 and below.

H means "hypertrophy", which denotes spleen hypertrophy indices of more than 1

Table 15: AVERAGE SPLEEN HYPERTROPHY INDICES FOR CN 180 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Spleen hypertrophy index
1st challenge: 4 weeks post-vaccination	
1	NH
2	H
3	H
6	H
2nd challenge: 8 weeks post-vaccination	
1	NH
2	NH
3	H
6	H
3rd challenge: 13 weeks post-vaccination	
1	NH
2	NH
5	H
6	H
4th challenge: 17 weeks post-vaccination	
4	NH
6	H
10	H
12	NH

Key NH means "no hypertrophy", which denotes spleen hypertrophy indices of 1 and below.

H means "hypertrophy", which denotes spleen hypertrophy indices of more than 1

Table 16: AVERAGE SPLEEN HYPERTROPHY INDICES FOR L46 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Spleen hypertrophy index
1st challenge: 4 weeks post-vaccination	
1	NH
2	H
3	H
6	H
2nd challenge: 8 weeks post-vaccination	
1	NH
2	H
3	H
6	H
3rd challenge: 13 weeks post-vaccination	
1	NH
2	NH
5	H
6	H
4th challenge: 17 weeks post-vaccination	
4	NH
6	NH
10	NH
12	H

Key: NH means "no hypertrophy", which denotes spleen hypertrophy indices of 1 and below

H means "hypertrophy", which denotes spleen hypertrophy indices of more than 1

Table 17: AVERAGE BACTERIAL LIVER COUNTS FOR UNVACCINATED BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Liver counts
1	1.6×10^4 org/g
2	NG
3	9.6×10^3 org/g
6	500 org/g

Key : NG means "no growth"

Org/g means "organisms per gramme of tissue"

Table 18: AVERAGE BACTERIAL LIVER COUNTS FOR CN 180 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Liver counts
1st challenge: 4 weeks post-vaccination	
1	NG
2	NG
3	650 org/g
6	1.0×10^3 org/g
2nd challenge: 8 weeks post-vaccination	
1	2.2×10^3 org/g
2	NG
3	100 org/g
6	350 org/g
3rd challenge: 13 weeks post-vaccination	
1	NG
2	200 org/g
5	2.2×10^3 org/g
6	600 org/g
4th challenge: 17 weeks post-vaccination	
4	200 org/g
6	4.1×10^5 org/g
10	100 org/g
12	NG

Key: NG means "no growth"
org/g means "organisms per gramme of tissue"

Table 19: AVERAGE BACTERIAL LIVER COUNTS FOR L46 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	Liver counts
1st challenge: 4 weeks post-vaccination	
1	NG
2	NG
3	NG
6	$8.8\text{x}10^{3}$ org/g
2nd challenge: 8 weeks post-vaccination	
1	$1.0\text{x}10^{3}$ org/g
2	$2.5\text{x}10^{4}$ org/g
3	NG
6	100 org/g
3rd challenge: 13 weeks post-vaccination	
1	100 org/g
2	NG
5	600 org/g
6	200 org/g
4th challenge: 17 weeks post-vaccination	
4	600 org/g
6	NG
10	NG
12	NG

Key: NG means "no growth"
org/g means "organisms per gramme of tissue"

The challenging doses for the second and third challenges were the same, i.e. 2,000 organisms per bird intraperitoneally, and yet the pattern of bacterial colonisation in the two challenges was different for both the L46 and CN 180 - vaccinated birds. With the second challenge, there was no time lag in both groups and the numbers on day 1 post-challenge were 2,200 organisms per gramme liver tissue (org/g) in CN 180 - vaccinated birds and 1000 org/g in L46 - vaccinated birds. This number fell to zero on day 2 post-challenge in CN 180 - vaccinated birds but rose to 25,700 org/g in L46 - vaccinated birds. On 3rd day post-challenge, the number rose to 100 org/g in CN 180 - vaccinated birds while that in L46 - vaccinated birds dropped to zero. On day 6 post-challenge, the number in CN 180 - vaccinated birds rose to 350 org/g while that in L46 - vaccinated birds rose to 100 org/g. With the third challenge, the maximum number of bacteria in the liver was 2,200 for CN 180 - vaccinated birds and 600 for L46 - vaccinated birds, this number being reached on day 5 post-challenge in both cases.

Eighty thousand (80,000) organisms were injected intraperitoneally per bird in the 4th challenge. Monitoring liver counts in CN 180 - vaccinated birds, it was 200 org/g on day 4 post-challenge, 410,000 org/g on day 6 post-challenge, 100 org/g on day 10 post-challenge and zero on day 12 post-challenge. Liver counts in L46 - vaccinated birds were 600 org/g on day 4 post-challenge and zero for the remaining days 6, 10 and 12 post-challenge.

The statistical comparison of (i) liver hypertrophy and resistance to bacterial multiplication in the liver and (ii) spleen hypertrophy and resistance to bacterial multiplication in the liver; as well as comparison of the ability of spleen and liver hypertrophies to detect no bacterial growths in the respective livers are given in Appendices 4, 5 and 6, respectively. The statistical results show that except for spleen hypertrophy in L46 - vaccinated birds, there was no significant difference between liver/spleen hypertrophy and resistance to bacterial multiplication as indicated by bacterial localisation in the liver (at 5% level of significance). In L46 - vaccinated birds, recovery of bacteria from the respective livers gave a better indication of resistance to bacterial multiplication than spleen hypertrophy. There was no significant differences between liver hypertrophy and spleen hypertrophy in relation to there being no bacterial growth in the respective livers.

The MIT and serological results for the controls, birds vaccinated with CN 180, and birds vaccinated with L46 were as given in Tables 20, 21, 22, 23, 24, 25, 26, 27 and 28, respectively. Figures 4 and 5 give the graphical representations of the MIT and IHA for the two vaccinated groups of birds, respectively, taking average figures for individual challenges. Its relationship to the other observations is given in Appendix 3. Since different birds were used each time, the results don't show a continuous immunologic response. They, however, give an idea of the immune status of the birds at various intervals.

Table 20: AVERAGE MIT RESULTS FOR UNVACCINATED BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	% Migration inhibition
1	NI
2	NI
3	14.8
6	54.2

Key: MIT means "Macrophage migration inhibition test"

NI means "no inhibition"

Table 21: AVERAGE MIT RESULTS FOR CN 180 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	% Migration inhibition
1st challenge: 4 weeks post-vaccination	
1	NI
2	1.85
3	43.2
6	57.7
2nd challenge: 8 weeks post-vaccination	
1	90.3
2	74.8
3	87.2
6	82.1
3rd challenge: 13 weeks post-vaccination	
1	-
2	-
5	-
6	40.9
4th challenge: 17 weeks post-vaccination	
4	29.13
6	33.2
10	42.47
12	40.9

Key: MIT means "Macrophage migration inhibition test"

- means "not done"

NI means "no inhibition"

Table 22: AVERAGE MIT RESULTS FOR L46 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	% Migration inhibition
1st challenge: 4 weeks post-vaccination	
1	15.5
2	27.6
3	30.2
6	52.3
2nd challenge: 8 weeks post-vaccination	
1	85.6
2	85.1
3	74.5
6	83.0
3rd challenge: 13 weeks post-vaccination	
1	-
2	-
5	-
6	48.9
4th challenge: 17 weeks post-vaccination	
4	26.0
6	51.4
10	48.85
12	68.85

Key: MIT means "Macrophage migration inhibition test"
- means "not done"
NI means "no inhibition"

RE 4

AVERAGE POOLED MIT RESULTS PER CHALLENGE IN PULLETS

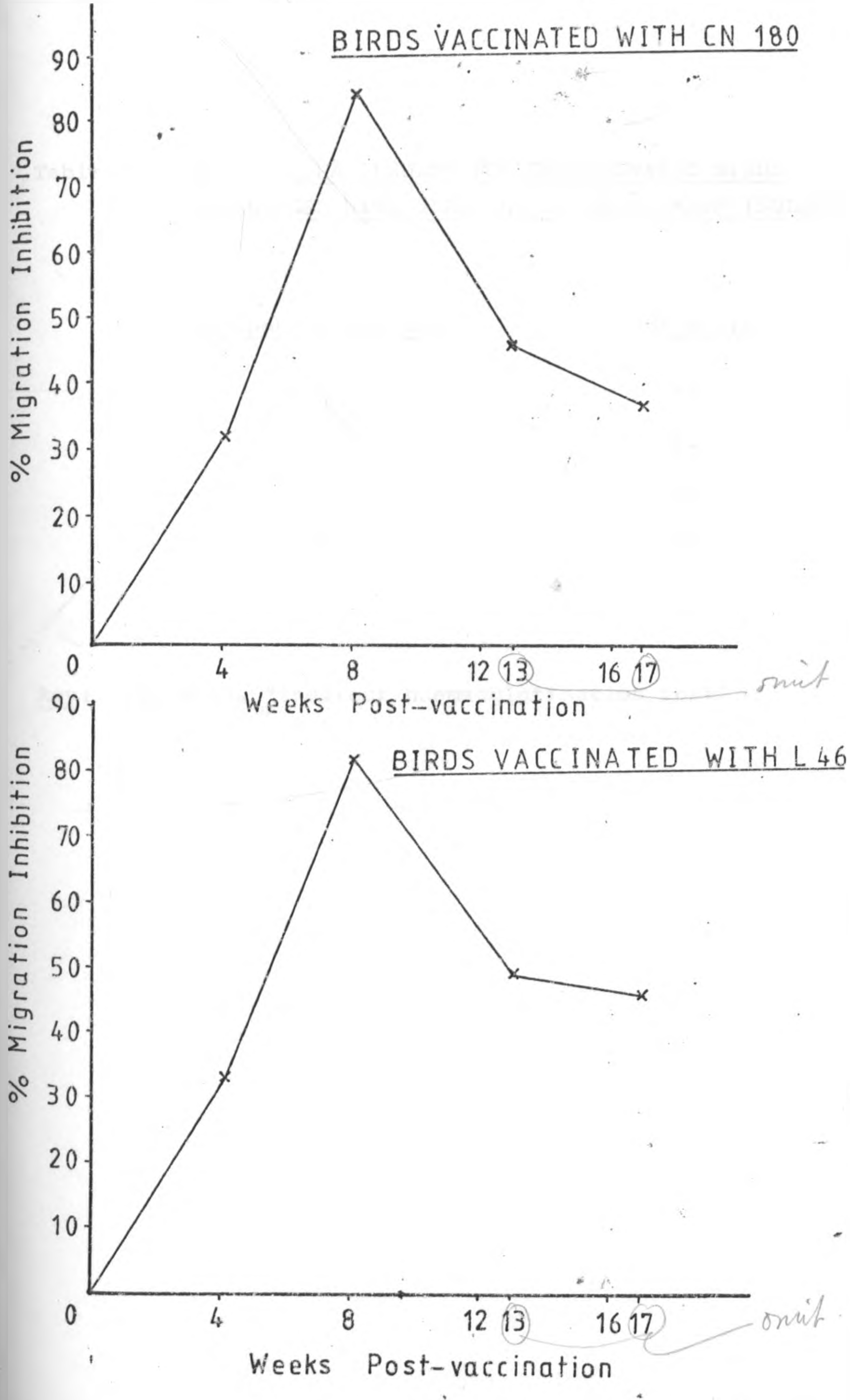


Table 23: AVERAGE IHA RESULTS FOR UNVACCINATED BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	IHA titre
1	64
2	64
3	64
6	64

Key: IHA means "indirect haemagglutination test"

Table 24: AVERAGE IHA RESULTS FOR CN 180 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	IHA titre
1st challenge: 4 weeks post-vaccination	
1	80
2	384
3	384
6	64
2nd challenge: 8 weeks post-vaccination	
1	96
2	288
3	128
6	256
3rd challenge: 13 weeks post-vaccination	
1	96
2	256
5	384
6	320
4th challenge: 17 weeks post-vaccination	
4	1,536
6	2,080
10	36,864
12	4,352

Key: IHA means "Indirect haemagglutination test"

Table 25: AVERAGE IHA RESULTS FOR L46 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	IHA titre
1st challenge: 4 weeks post-vaccination	
1	512
2	288
3	2,176
6	8,208
2nd challenge: 8 weeks post-vaccination	
1	96
2	160
3	2,080
6	128
3rd challenge: 13 weeks post-vaccination	
1	192
2	48
5	256
6	512
4th challenge: 17 weeks post-vaccination	
4	192
6	256
10	1,280
12	4,096

Key: IHA means "Indirect haemagglutination test"

FIGURE 5

AVERAGE POOLED IHA TITRES PER CHALLENGE IN PULLETS

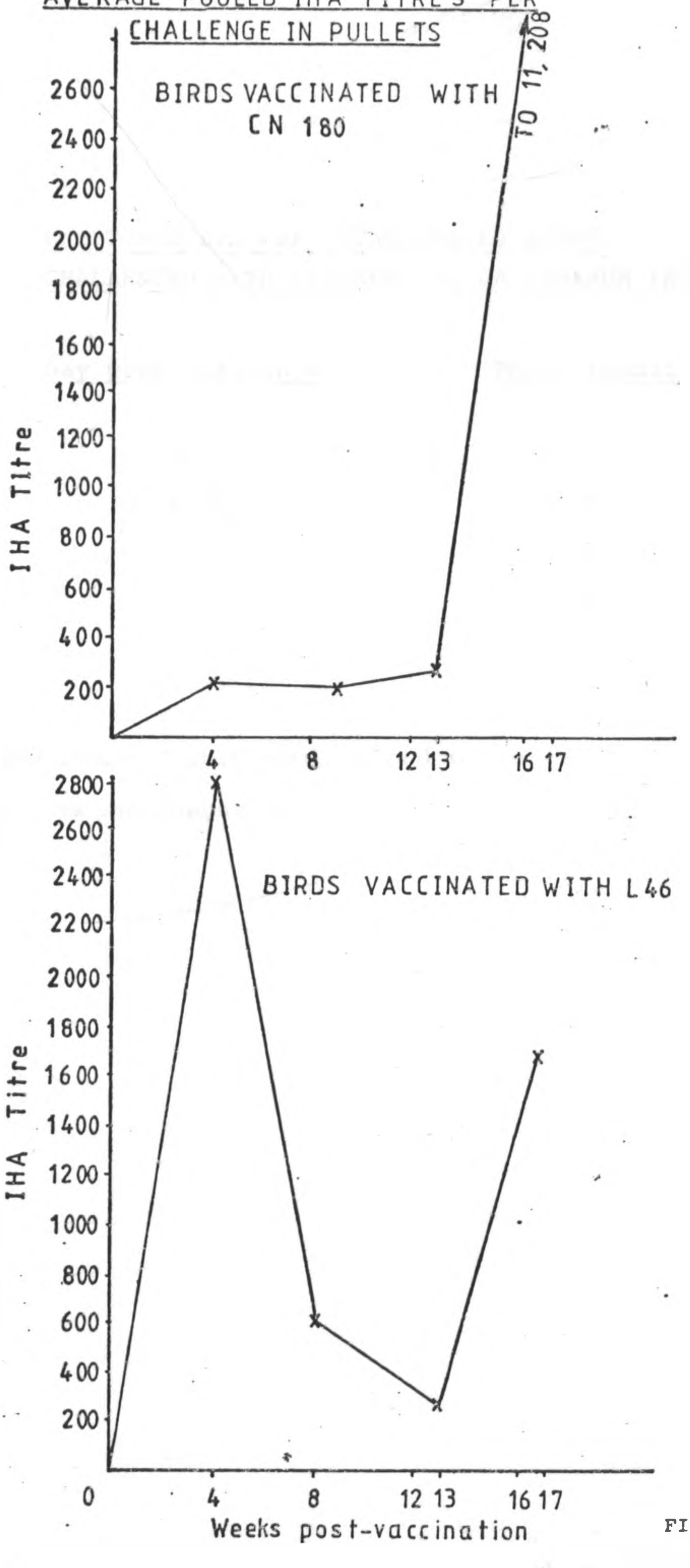


FIGURE 5

Table 26: RWBPT RESULTS FOR UNVACCINATED BIRDS CHALLENGED WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	RWBPT result
1	-ve
2	-ve
3	-ve
6	-ve

Key: RWBPT means "Rapid whole blood plate test"

-ve means "negative"

Table 27: RWBPT RESULTS FOR CN-180-VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	RWBPT Result
1st challenge: 4 weeks post-vaccination	
1	+ve
2	+ve(-ve)
3	+ve(-ve)
6	-ve
2nd challenge: 8 weeks post-vaccination	
1	-ve
2	+ve
3	+ve
6	+ve
3rd challenge: 13 weeks post-vaccination	
1	+ve(-ve)
2	+ve(-ve)
5	+ve
6	+ve(-ve)
4th challenge: 17 weeks post-vaccination	
4	-ve
6	+ve
10	+ve
12	+ve

Key RWBPT means "Rapid whole blood plate test"
+ve means "positive"
-ve means "negative"
(-ve) means "one of the 2 birds gave negative reaction"

Table 28: RWBPT RESULTS FOR L46 - VACCINATED BIRDS CHALLENGED AT VARIOUS INTERVALS WITH VIRULENT S. GALLINARUM ISOLATE

Day post-challenge	RWBPT Result
1st challenge: 4 weeks post-vaccination	
1	+ve
2	+ve
3	+ve
6	+ve (-ve)
2nd challenge: 8 weeks post-vaccination	
1	-ve
2	+ve
3	+ve (-ve)
6	+ve (-ve)
3rd challenge: 13 weeks post-vaccination	
1	+ve (-ve)
2	-ve
5	+ve
6	+ve
4th challenge: 17 weeks post-vaccination	
4	+ve (-ve)
6	+ve
10	+ve
12	+ve

Key: RWBPT means "Rapid whole blood plate test"
+ve means "positive"
-ve means "negative"
(-ve) means "one of the 2 birds gave negative reaction"

The results showed that both CN 180 and L46 - vaccinated birds followed a similar immune reaction when monitored by MIT. The highest macrophage migration inhibition was shown in birds used for the second challenge (8 weeks post-vaccination), the degree of inhibition reducing in the third (13 weeks post-vaccination) and fourth (17 weeks post-vaccination) challenges. However, there was a marked difference in their IHA responses. The highest IHA titre for CN 180 - vaccine was 11,208 which was detected 17 weeks post-vaccination. The other titres were 228, 192 and 264, detected at 4, 8 and 13 weeks post-vaccination. The highest titre for L46 - vaccine was 2,800 at 4 weeks post-vaccination. The other titres were 616, 252 and 1664 at 8, 13 and 17 weeks post-vaccination, respectively.

There was no macrophage migration inhibition in most of the control birds screened as shown in Table 20. It is, however, interesting to note that the two challenged control birds, which were sacrificed on day 6 post-challenge had macrophage migration inhibitions of 49.05% and 59.37% respectively (average inhibition 54.2%). Similarly, the control birds showed low IHA titres, averaging to 54 overall (Table 23). They also gave negative reaction to RWBPT.

4.8 ANTIBIOTIC SENSITIVITY TESTING OF THE S. GALLINARUM ISOLATES

Thirty-five (35) of the *S. gallinarum* isolates were subjected to 14 common antibiotics in *in vitro* tests. The results were as shown in Table 29 and figures 6, 7 and 8. The detailed results are given in Appendix 7.

From Figure 6, it is evident that the seven antibiotics that were most effective (giving 100% kill) were Nitrofurantoin, Gentamycin, chloramphenicol, Tetracycline, Ampicillin, furazolidone and Neomycin. The rest ranked in a decreasing order as shown:- Cotrimoxazole and Erythromycin (97%), Nalidixic acid (87%), Streptomycin (43%) and compound sulphur (31%). All isolates were resistant to cloxacillin and penicillin. There was great diversity in susceptibility to the antibiotics tested (Appendix 7).

There was marked multiple resistance to antibiotics (Figure 8):- 3% to 2 antibiotics, 23% to 3 antibiotics, 26% to 4 antibiotics, 40% to 5 antibiotics, 6% to 6 antibiotics and 3% to over 7 antibiotics (actually it was resistant 10 antibiotics).

The type cultures, *E. coli* and *St. aureus* showed that the penicillin-G and cloxacillin were effective on gram-positive bacteria and not gram-negative ones.

Table 29: SUSCEPTIBILITY OF S. GALLINARUM ISOLATES TO 14 COMMON ANTIBIOTICS

Antibiotic	Number susceptible	Number resistant
Co-trimoxazole (25 μg)	29	1
Nitrofurantoin (200 μg)	30	0
Nalidixic acid (30 μg)	26	4
Gentamycin (30 μg)	30	0
Chloramphenicol (10 μg)	35	0
Compound Sulphonamide (200 μg)	11	24
Tetracycline (100 μg)	30	0
Erythromycin (10 μg)	29	1
Ampicillin (25 μg)	30	0
Cloxacillin (5 μg)	0	30
Penicillin G. (1.5 i.u)	0	35
Streptomycin (25 μg)	13	17
Furazolidone (15 μg)	9	0
Neomycin (10 μg)	9	0

NB : The figures in parenthesis denote the respective antibiotic concentrations contained in each disc.

FIGURE 6

KEY

SXT - Cotrimoxazole (25 μg)

F - Nitrofurantoin (200 μg)

NA - Nalidixic acid (30 μg)

CN - Gentamycin (10 μg)

C - Chloramphenicol (30 μg)

S3 - Compound Sulphur (200 μg)

Te - Tetracycline (100 μg)

E - Erythromycin (10 μg)

AMP - Ampicillin (25 μg)

OB - Cloxacillin (5 μg)

P - Penicillin G (1.5 i.u.)

S - Streptomycin (25 μg)

FR - Furazolidone (15 μg)

N - Neomycin (10 μg)

6

SUSCEPTIBILITY (PERCENT) OF S. GALLINARUM TO 14 COMMON ANTIBIOTICS.

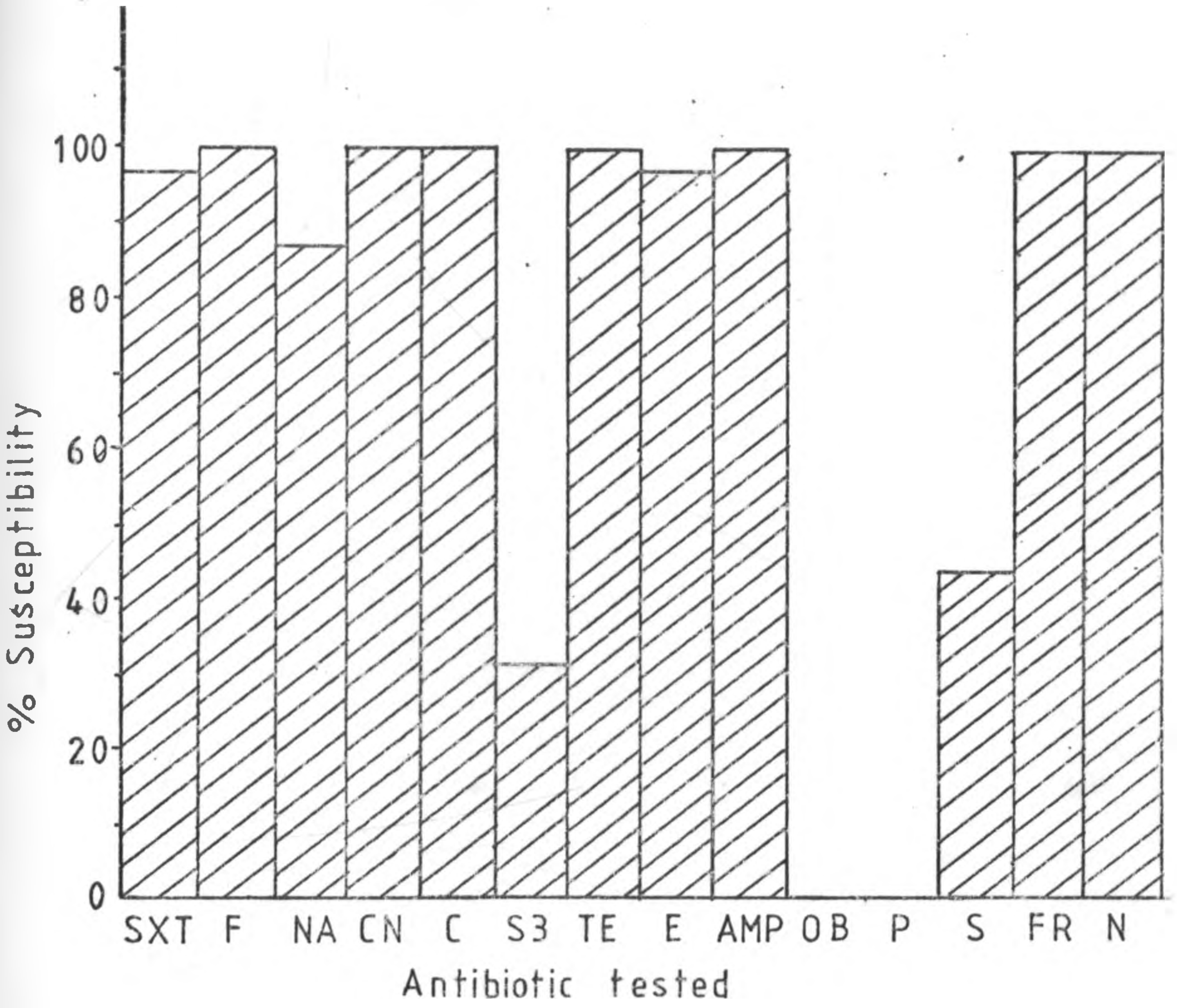


FIGURE 6

FIGURE 7. ANTIBIOTIC SENSITIVITY PROFILES FOR ONE S. GALLINARUM ISOLATE SHOWING DIVERSE SENSITIVITY REACTIONS SHOWN BY VARIOUS BACTERIA.

FREQUENCY OF MULTIPLE RESISTANCE TO TESTED ANTIBIOTICS AMONG S.GALLINARUM ISOLATES

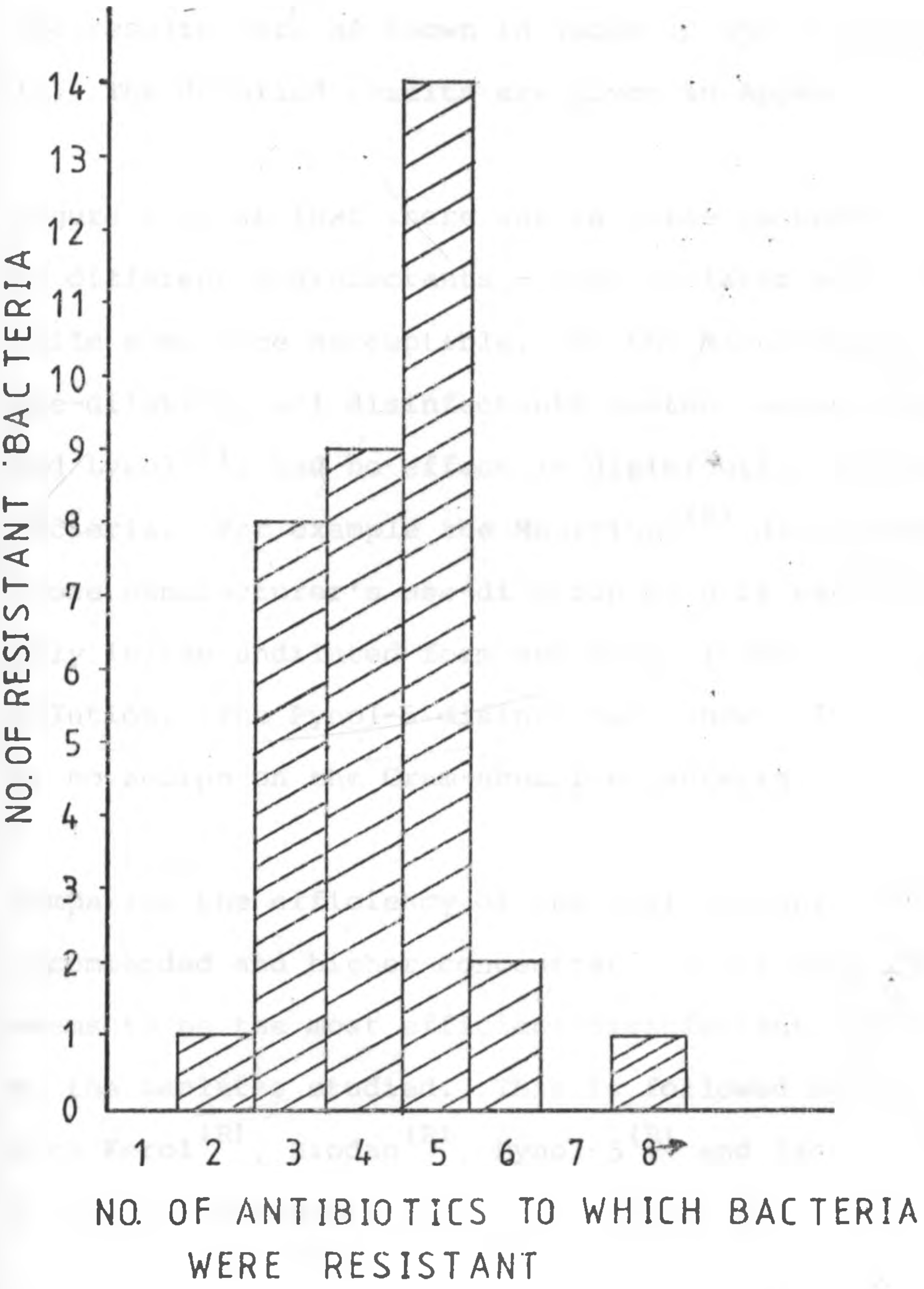


FIGURE 8

4.9 DISINFECTANT SENSITIVITY TESTING OF THE VARIOUS S. GALLINARUM ISOLATES

Thirty (30) of the *S. gallinarum* isolates were subjected to 6 common disinfectants in *in vitro* tests. The results were as shown in Table 30 and Figures 9 and 10. The detailed results are given in Appendix 8.

Figure 9 shows that there was variable bacterial susceptibility to different disinfectants - some isolates were resistant while some were susceptible. At the manufacturer's use-dilution, all disinfectants tested, except Bromosept$^{(R)}$ and Lysol$^{(R)}$, had no effect in disinfection of the *Salmonella* bacteria. For example the Municipal$^{(R)}$ disinfectant fluid, whose manufacturer's use-dilution is 0.2% was effective only in the undiluted form and only slightly so in the 10% dilution. The Pynol-5 disinfectant showed limited activity or no action on the Gram-negative bacteria.

Comparing the efficiency of the disinfectants at the recommended and higher concentrations for use, Bromosept$^{(R)}$ seems to be the most efficient disinfectant, with regard to the isolates studied. This is followed by Lysol$^{(R)}$, then Kerol$^{(R)}$, Biodan$^{(R)}$, Pynol-5$^{(R)}$ and lastly Municipal fluid$^{(R)}$ (Figure 9).

Table 30: SUSCEPTIBILITY OF S. GALLINARUM ISOLATES TO 6 COMMON DISINFECTANTS

Disinfectant at Various concentations		Number susceptible	Number resistant
Lysol	-0.5%	0	30
	- 1%	0	30
	- 2%	12	18
	- 4%	30	0
Pynol	-5 - 1%	0	30
	- 2%	0	30
	- 5%	4	26
	-10%	9	21
Kerol	-0.17%	0	30
	-0.33%	0	30
	-1.0%	17	13
	-1.7%	27	3
	-3.3%	30	0
Biodan	-0.6%	0	30
	-1.8%	0	30
	-2.5%	1	29
	- 4%	19	11
	- 6%	29	1
Bromosept	-0.01%	24	6
	-0.1%	28	2
	-0.5%	29	1
	- 1%	30	0
	- 2%	30	0
Municipal fluid	-0.3%	0	30
	-0.7%	0	30
	- 2%	0	30
	-10%	3	27
	-100%	30	0

FIGURE 9:

<u>NB</u>

The dilutions marked with asterix are the use-dilutions recommended by the respective manufacturers.

FIGURE 9 SUSCEPTIBILITY (PERCENT) OF S.GALLINARUM ISOLATES TO 6 COMMON DISINFECTANTS

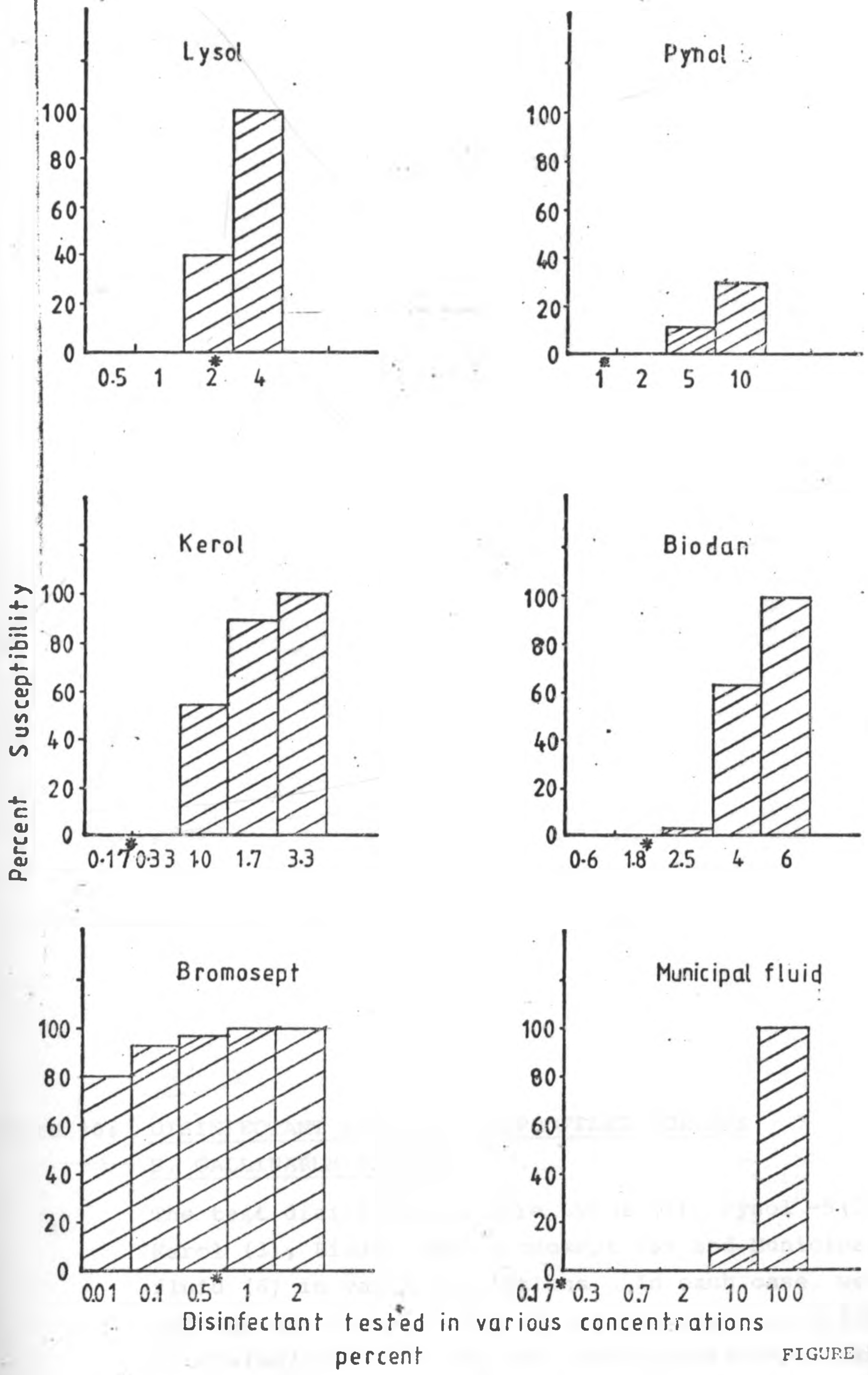


FIGURE 9

Figure 10: <u>DISINFECTANT SENSITIVITY PROFILES FOR ONE S. GALLINARUM ISOLATE</u>

The test disinfectants were Lysol (1), Pynol -5(2), Kerol (3), Biodan (4), Bromosept (5) and Municipal fluid (6) in various dilutions. In each case, well 'a' had the lowest dilution, with increasing dilution clockwise; well 'o' was the control with distilled water. The medium used was oxoid isosensitest agar.

Of the type (control) cultures processed, *E.coli* gave more-or-less the same reactions as the *S. gallinarum* isolates. *Staph. aureus* gave similar reactions as the *S. gallinarum* isolates when tested with Lysol$^{(R)}$, Biodan$^{(R)}$ and Bromosept$^{(R)}$, but showed greater sensitivity to Pynol and Kerol, both at recommended and higher concentrations.

4.10 PHAGE TYPING OF S. GALLINARUM ISOLATES

The results of phage-typing on the 67 *S. gallinarum* isolates are given in Table 31. There are variations in the degree of lysis shown by various isolates which indicates some differences in phage receptors on the various isolates. The isolates were most sensitive to phage 4 where 98.5% of them showed total lysis (ranging from 3+ to 4+) with only one (1) showing partial lysis of 2+. The next effective phages were phage 3 and phage 2 where 86.6% and 83.6% of the isolates, respectively, showed total lysis. Forty-three isolates (64.2%) showed total sensitivity to phage 5 while 34.3% showed partial sensitivity. The isolates had the least sensitivity to phage 1 where 62.7% of them showed partial lysis and only 35% showed total lysis. Isolate L1 showed no sensitivity to phage 1, and isolate L15 showed no sensitivity to phages 2 and 5.

Table 31: PHAGE TYPING OF S. GALLINARUM ISOLATES USING FIVE DIFFERENT PHAGES

Isolate	Phage 1	Phage 2	Phage 3	Phage 4	Phage 5
L1	-ve	3+	1+	3+	4+
L2	1+	1+	3+	2+	1+
L3	1+	2+	3+	3+	1+
L4	1+	2+	4+	3+	3+
L5	1+	3+	1+	4+	3+
L6	1+	3+	2+	3+	3+
L7	1+	3+	2+	4+	2+
L8	1+	3+	2+	4+	3+
L9	1+	3+	3+	3+	2+
L10	1+	3+	3+	3+	3+
L11	1+	3+	3+	3+	3+
L12	1+	3+	3+	4+	3+
L13	1+	3+	3+	4+	3+
L14	1+	3+	4+	3+	2+
L15	2+	-ve	4+	4+	-ve
L16	2+	2+	2+	4+	2+
L17	2+	2+	3+	3+	3+
L18	2+	2+	3+	3+	3+
L19	2+	2+	4+	3+	2+
L20	2+	3+	2+	3+	3+
L21	2+	3+	2+	4+	3+
L22	2+	3+	3+	3+	3+
L23	2+	3+	3+	3+	3+
L24	2+	3+	3+	3+	3+
L25	2+	3+	3+	3+	3+
L26	2+	3+	3+	3+	3+
L27	2+	3+	3+	3+	3+
L28	2+	3+	3+	3+	3+
L29	2+	3+	3+	4+	3+
L30	2+	3+	3+	4+	3+
L31	2+	3+	3+	4+	3+
L32	2+	3+	3+	4+	3+

Table 31 Cont......

Isolate	Phage 1	Phage 2	Phage 3	Phage 4	Phage 5
L33	2+	3+	4+	3+	3+
L34	2+	3+	3+	4+	4+
L35	2+	3+	4+	4+	3+
L36	2+	4+	2+	4+	2+
L37	2+	4+	3+	3+	2+
L38	2+	4+	3+	3+	3+
L39	2+	4+	3+	4+	3+
L40	2+	4+	4+	3+	1+
L41	2+	4+	4+	3+	2+
L42	2+	4+	4+	3+	2+
L43	2+	4+	4+	3+	3+
L44	3+	1+	3+	4+	2+
L45	3+	2+	3+	4+	2+
L46	3+	3+	3+	3+	2+
L47	3+	3+	3+	3+	2+
L48	3+	3+	3+	3+	2+
L49	3+	3+	3+	3+	2+
L50	3+	3+	3+	3+	3+
L51	3+	3+	3+	3+	3+
L52	3+	3+	3+	3+	3+
L53	3+	3+	3+	3+	3+
L54	3+	3+	3+	3+	3+
L55	3+	3+	3+	4+	2+
L56	3+	3+	4+	3+	2+
L57	3+	3+	4+	3+	3+
L58	3+	4+	3+	3+	3+
L59	3+	4+	4+	3+	2+
L60	3+	4+	4+	3+	4+
CN 180	3+	4+	4+	4+	3+
L62	3+	4+	4+	4+	3+
L63	4+	1+	4+	4+	1+
L64	4+	3+	3+	3+	3+
L65	4+	3+	4+	3+	2+
L66	4+	3+	4+	3+	3+
L67	4+	3+	4+	4+	3+

Key: 1+, 2+, 3+ and 4+ are degrees of lysis as shown by clearing. Complete clearing was assigned the value of 4+ and faint clearing a value of 1+

4.11 A COMPARATIVE STUDY OF THE VARIOUS S. GALLINARUM SOMATIC ANTIGENS IN RELATION TO THE ANTIBODY TITRES THEY ELICIT

When sera from birds suffering from fowl typhoid, as well as vaccinated ones, were screened for *Salmonella* antibody titres using different *S. gallinarum* somatic antigens, the results were as shown in Table 32. Statistical analysis of the ability of one antigen to produce higher titres than the others is given in Appendix 9. The results showed that, except for antigens 1 and 12, there was no significant difference in the frequencies at which one antigen showed higher titres than the other two. The frequency at which antigen 1 showed higher titres than antigen 9 and 12 was higher than at which antigen 12 showed higher titres than antigens 1 and 9.

The titres were not the same for all the three antigens used, for any one serum, and the peak titres, per serum, in the majority of the cases, varied from one antigen to the other. This indicates that the causative agents had varying quantities of the three antigens, respectively, and that there is a possibility that there were more than one "strain" of *S. gallinarum* involved. It is interesting to note that, in the majority of the cases, titres to the pooled antigens (factors) were lower than those to individual antigen(s).

Table 32: IHA TITRES TO THE VARIOUS S. GALLINARUM ANTIGENS, AS SHOWN BY SERA FROM VARIOUS FLOCKS OF BIRDS (FARMS) SUFFERING FROM FOWL TYPHOID

Farm No.	Specimen No.	Factor 1	Factor 9	Factor 12	Pooled Factors
1	1	8	256	128	$1x10^3$
	2	128	128	128	64
	3	64	64	32	64
	4	128	64	128	256
	5	64	128	32	128
	6	256	64	256	64
	7	128	128	32	128
	8	512	256	256	64
	9	128	64	64	64
	10	64	32	32	64
	11	16	16	16	32
2	1	$1.6x10^4$	$6.5x10^4$	$4.1x10^3$	512
	2	$3.2x10^4$	$6.6x10^4$	$1.6x10^4$	$6.6x10^4$
	3	$2.0x10^3$	$8.2x10^3$	$8.2x10^3$	$8.2x10^3$
	4	$8.2x10^3$	$8.2x10^3$	$6.6x10^4$	$8.2x10^3$
	5	$8.2x10^3$	$1.6x10^4$	$1.6x10^4$	$1.6x10^4$
	6	$8.2x10^3$	$1.6x10^4$	$4.1x10^3$	512
	7	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$	$3.3x10^4$
3	1	$1.6x10^4$	$6.6x10^4$	$3.3x10^4$	$8.2x10^3$
	2	$6.6x10^4$	$1.6x10^4$	$6.6x10^4$	$8.2x10^3$
	3	$1.6x10^4$	$1.6x10^4$	$8.2x10^3$	$4.1x10^3$
	4	$6.6x10^4$	$1.6x10^4$	$3.3x10^4$	$8.2x10^3$
	5	$8.2x10^3$	$8.2x10^3$	$6.6x10^4$	$8.2x10^3$
	6	$6.6x10^4$	$1.6x10^4$	$1.6x10^4$	$8.2x10^3$
	7	$8.2x10^3$	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$
	8	$1.6x10^4$	$6.6x10^4$	$1.6x10^4$	$1.6x10^4$
	9	$8.2x10^3$	$1.6x10^4$	$3.3x10^4$	$8.2x10^3$
	10	$8.2x10^3$	$8.2x10^3$	$4.1x10^3$	512

Table 32 Cont....

Farm No.	Specimen No.	Factor 1	Factor 9	Factor 12	Pooled Factors
4	1	$8.2x10^3$	$8.2x10^3$	$8.2x10^3$	$8.2x10^3$
	2	$6.6x10^4$	$1.6x10^4$	$6.6x10^4$	$8.2x10^3$
	3	$6.6x10^4$	$1.6x10^4$	$6.6x10^4$	$6.6x10^4$
	4	$3.3x10^4$	$1.6x10^4$	$8.2x10^3$	$8.2x10^3$
	5	$6.6x10^4$	$1.6x10^4$	$1.6x10^4$	$6.6x10^4$
	6	$1.6x10^4$	$1.6x10^4$	$1.0x10^3$	$1.0x10^3$
	7	$1.6x10^4$	$8.2x10^3$	$6.6x10^4$	$8.2x10^3$
5	1	$6.6x10^4$	$4.1x10^3$	$1.0x10^3$	$4.1x10^3$
	2	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$
	3	$6.6x10^4$	$6.6x10^4$	$8.2x10^3$	$1.6x10^4$
	4	$6.6x10^4$	$1.0x10^3$	$4.1x10^3$	$1.0x10^3$
	5	$8.2x10^3$	512	$8.2x10^3$	512
	6	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$
	7	$3.3x10^4$	$3.3x10^4$	$6.6x10^4$	256
	8	$1.6x10^4$	$1.6x10^4$	$1.6x10^4$	$4.1x10^3$
	9	$8.2x10^3$	$8.2x10^3$	$4.1x10^3$	512
	10	$6.6x10^4$	$4.1x10^3$	$4.1x10^3$	512
6	1	$6.6x10^4$	$1.6x10^4$	$8.2x10^3$	$8.2x10^3$
	2	$6.6x10^4$	$6.6x10^4$	$3.3x10^4$	$6.6x10^4$
	3	$8.2x10^3$	$4.1x10^3$	$2.0x10^3$	512
	4	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$
	5	$1.6x10^4$	$1.6x10^4$	$1.6x10^4$	$6.6x10^4$
	6	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$
	7	$6.6x10^4$	$6.6x10^4$	$1.6x10^4$	$6.6x10^4$
	8	$6.6x10^4$	$1.6x10^4$	$8.2x10^3$	$8.2x10^3$
	9	$3.3x10^4$	$8.2x10^3$	$1.0x10^3$	$2.0x10^3$
	10	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$	$6.6x10^4$
	11	$6.6x10^4$	$6.6x10^4$	$8.2x10^3$	$3.3x10^4$

Key Farm Number 1 consisted of sera from vaccinated birds in the field. They had completed their term of lay and were being slaughtered for sale. They had no history of fowl-typhoid disease.

Farms numbered 2, 3, 4, 5 and 6 consisted of sera from naturally infected birds that had an outbreak of fowl-

4.12 HUMORAL AND CELLULAR IMMUNITY TO THE DIFFERENT S. GALLINARUM SOMATIC ANTIGENS AS ASSAYED IN EXPERIMENTAL COCKERELS

The results of SAT, TAT and IHA serological tests, with regard to the 4 antigens used were as given in Tables 33 and 34. Figures 11 and 12 represent the TAT and IHA titres, respectively.

In general, the TAT antibody titres to *Salmonella* antigens 9 (*S. strasbourg*) and 12 (S. *Kiambu*) were higher than that to *Salmonella* antigen 1 (*S. senftenberg*), the peak titres being 640 for antigens 9 and 12 and 320 for antigen 1. The titre to the pooled antigens 1, 9 and 12 (*S. gallinarum*) was also low, the peak titre being 320. In all cases, the titres fluctuated a lot as the experiment progressed. For *Salmonella* antigen 1, the peak titre was detected on day 3 post-vaccination. For *Salmonella* antigens 9 and 12, the peak titres were detected on day 19 post-vaccination. That for the pooled antigen was detected on days 19 and 39 post-vaccination. The birds that were suffering from natural fowl-typhoid infection (designated as I above) gave the same titre of 320 for the 3 different *Salmonella* antigens and a titre of 640 for the pooled antigen.

The control birds showed titres as high as 320 on days 3 and 39 post-vaccination to *Salmonella* antigen 12; on day 19 to the pooled antigen; and on day 39 to antigen 9.

Table 33: ANTIBODY TITRES TO S. GALLINARUM SOMATIC ANTIGENS IN EXPERIMENTALLY - VACCINATED BIRDS

Day Post-Vaccination		Slide agglutination				RWBPT	Tube agglutination				IHA titre
		Ag1	Ag2	Ag3	Ag4		Ag1	Ag2	Ag3	Ag4	
3	T	+	+	+	+	+	160	320	160	320	256
	C	+	+	+	+	+	320	160	160	320	256
6	T	+	+	+	+	+	160	160	160	160	512
	C	+	+	+	+	+	160	80	160	160	128
10	T	+	+	+	+	+	320	160	160	320	128
	C	+	+	+	+	+	80	80	80	80	256
13	T	+	+	+	+	-	160	160	80	160	128
	C	+	+	+	+	+	80	40	80	80	16
19	T	+	+	+	+	+	640	80	320	640	256
	C	+	+	+	+	+	160	160	320	160	128
26	T	+	+	+	+	+	320	160	160	320	128
	C	+	+	+	+	+	80	160	80	80	64
32	T	+	+	+	+	-	80	80	80	80	128
	C	+	+	+	+	+	160	160	160	160	64
39	T	+	+	+	+	-	160	160	320	160	128
	C	+	+	+	+	+	320	160	160	320	32
46	T	+	+	+	+	+	160	160	160	320	128
	C	+	+	+	+	+	80	80	80	80	64
I		+	+	+	+	+	320	320	640	320	1024

Key

+ means "positive i.e. agglutination"

- means "negative i.e. no agglutination"

T means "test birds"

C means "control birds"

I means "clinically infected birds from the field"

Ag1 means "S. Kiambu"

Ag2 means "S.senftenberg"

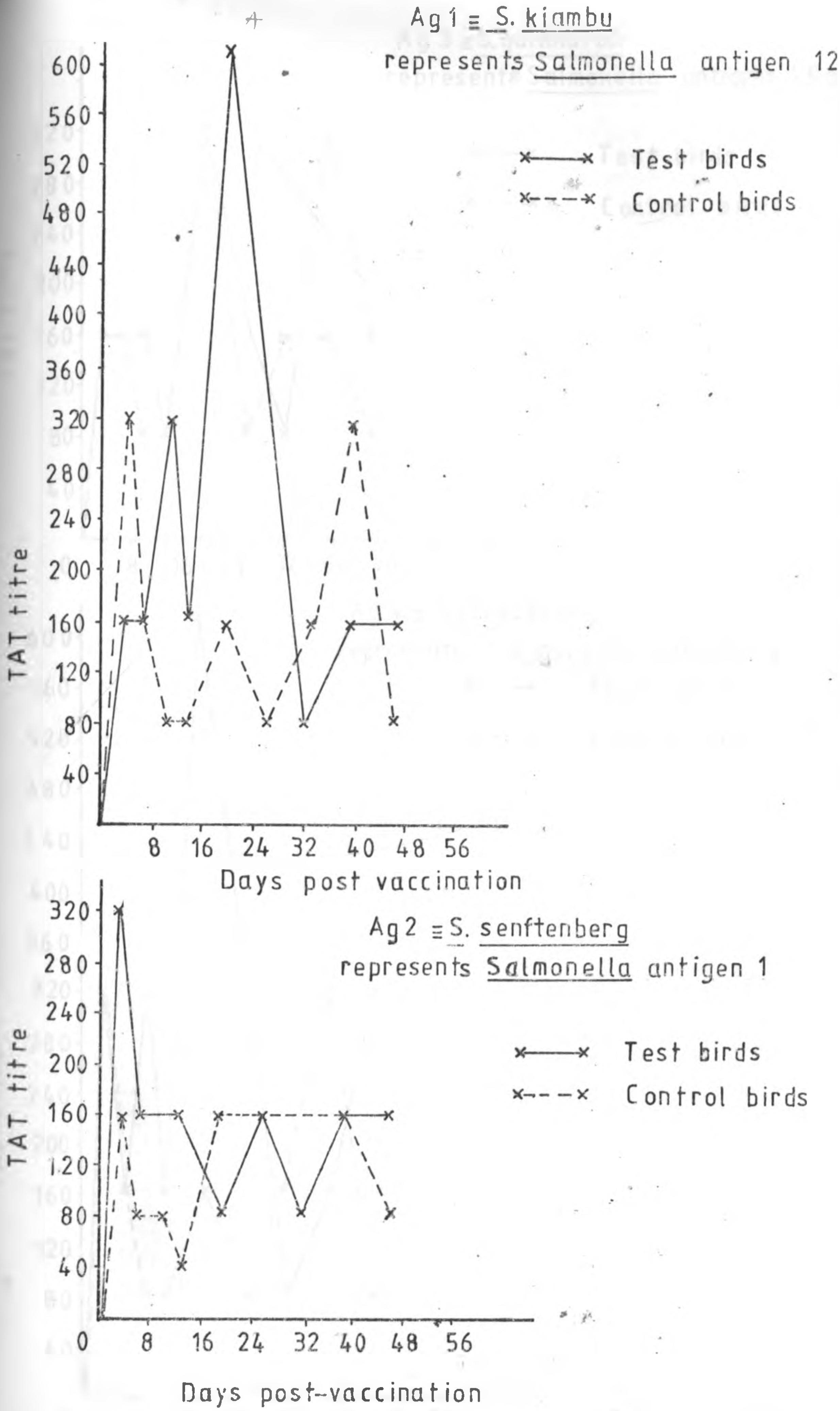
Ag3 means "S.gallinarum" strain L25

Ag4 means "S.strasbourg"

RWBPT means "rapid whole blood plate test"

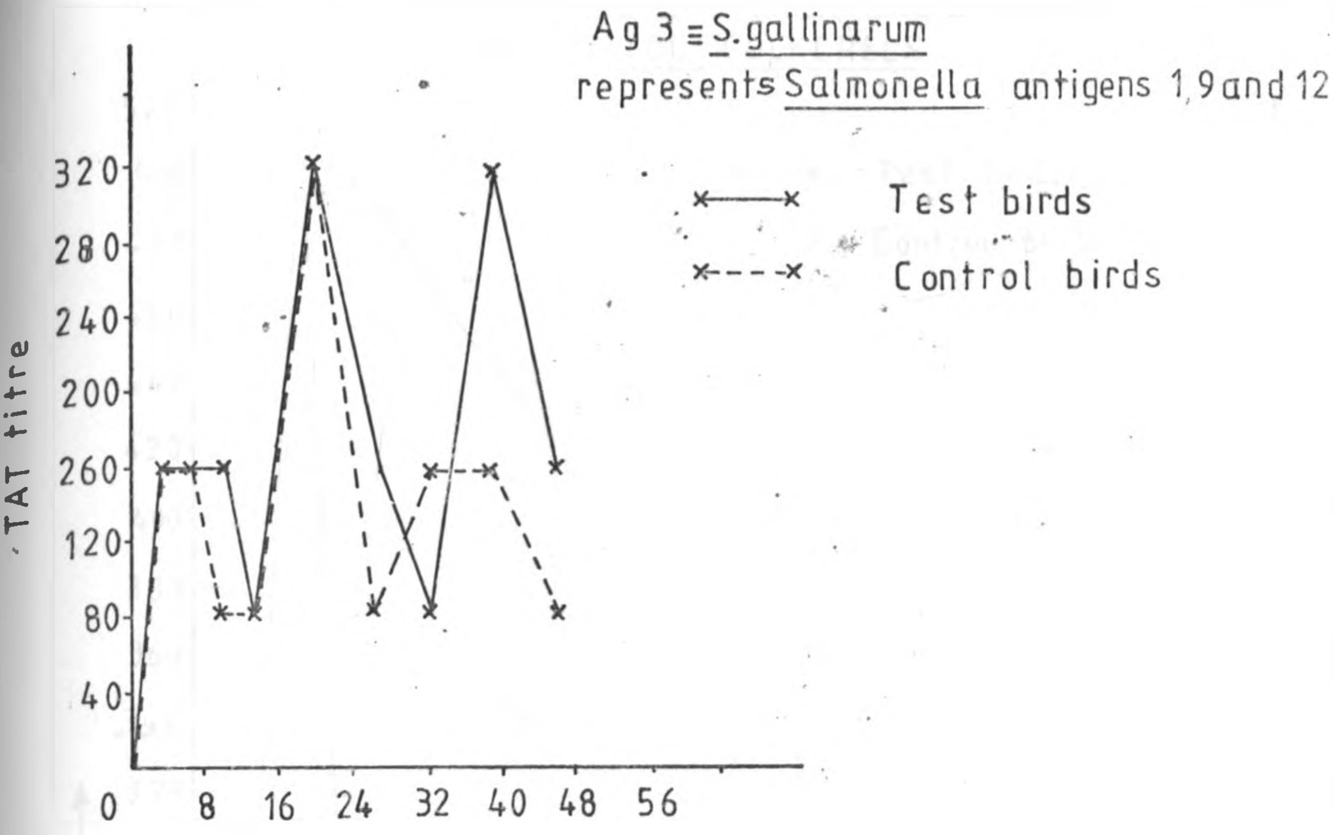
IHA means "indirect haemagglutination test"

FIGURE 11: TAT TITRES TO THE VARIOUS S. GALLINARUM SOMATIC ANTIGENS IN EXPERIMENTALLY-VACCINATED BIRDS

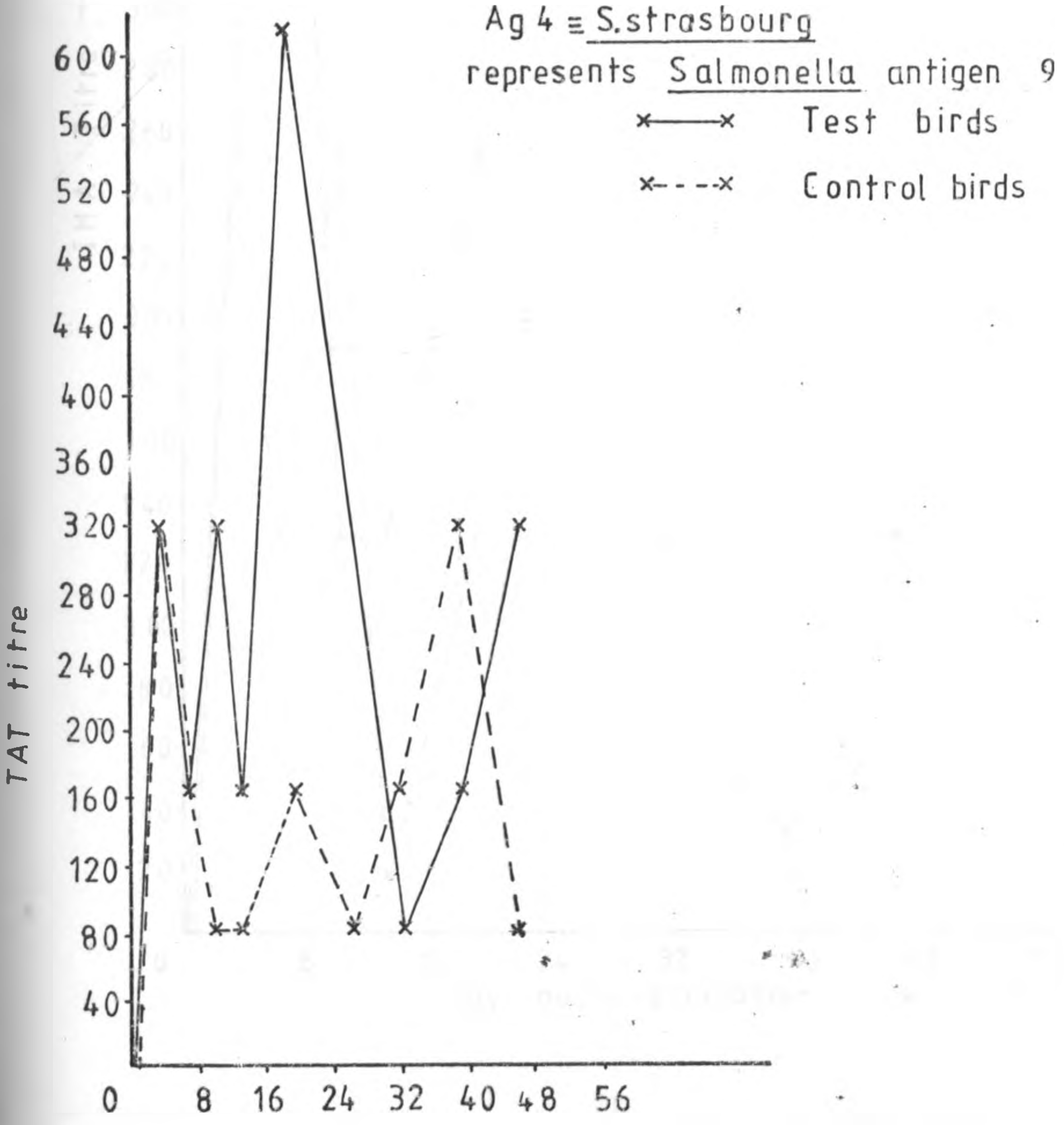

Ag 1 ≡ *S. kiambu*
represents *Salmonella* antigen 12

Ag 2 ≡ *S. senftenberg*
represents *Salmonella* antigen 1

FIGURE 11



Ag 3 ≡ *S. gallinarum*
represents *Salmonella* antigens 1,9 and 12



Ag 4 ≡ *S. strasbourg*
represents *Salmonella* antigen 9

FIGURE 12

IHA TITRES TO POOLED ANTIGEN (3) (S.GALLINARUM) IN EXPERIMENTALLY— VACCINATED COCKERELS

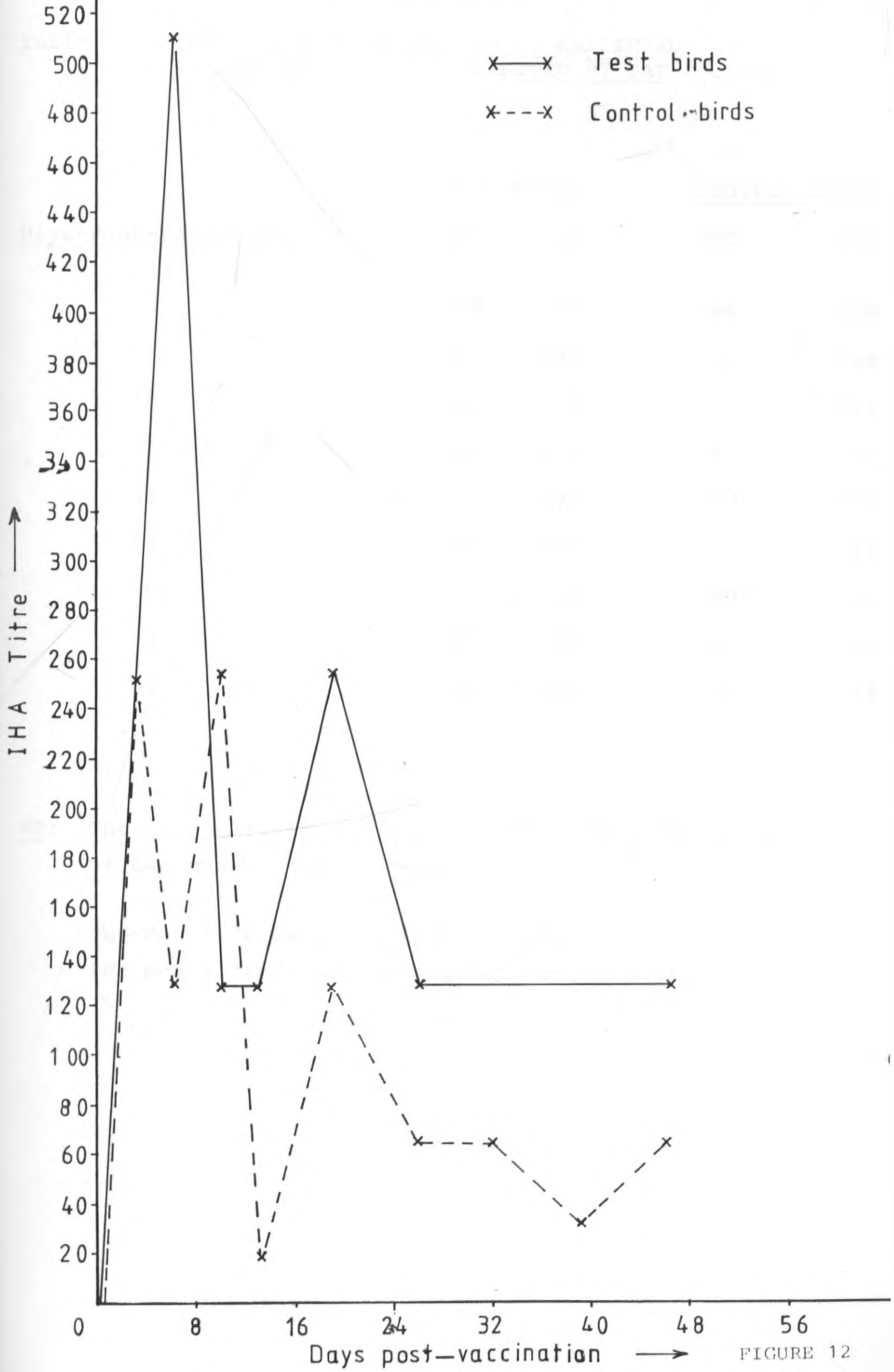


FIGURE 12

Table 34: COMPARISON OF TITRES TO S. GALLINARUM (POOLED ANTIGEN) AS DETECTED BY TAT AND IHA

	TEST BIRDS		CONTROL BIRDS	
Days-Post-Vaccination	TAT	IHA	TAT	IHA
3	160	256	160	256
6	160	512	160	128
10	160	128	80	256
13	80	128	80	16
19	320	256	320	128
26	160	128	80	64
32	80	128	160	64
39	320	128	160	32
46	160	128	80	64

NB: The naturally-infected birds (I) gave a TAT titre of 640 and an IHA titre of 1,024

TAT means "tube agglutination test"
IHA means "indirect haemagglutination test"

The highest titre to *Salmonella* antigen 1, in the control birds, was 160. It was detected on days 3, 19, 26, 32 and 39 post-vaccination. As in the test-birds, the titres fluctuated a lot as the experiment progressed.

The IHA titres to the pooled antigen also showed a lot of fluctuation both in the test and control birds. The highest titre in the test birds was 512 which was detected on day 6 post-vaccination, and that for the control birds was 256 detected on days 3 and 10 post-vaccination. The lowest titre in the test birds was 128 detected on days 10, 13, 26, 32, 39 and 46 post-vaccination. That for the control birds was 16 detected on day 13 post-vaccination. The naturally infected birds (I) showed a titre of 1,024. The SAT gave strong agglutination with all the 4 antigens and the RWBPT was positive for most of the screenings done.

Overall, the TAT has detected higher titres to *S. gallinarum* (pooled antigen) than the IHA (Table 34), although the peak titre for IHA was 512 while that for TAT was 320. The naturally-infected birds gave a titre of 1,024 with IHA and that of 640 with TAT.

The MIT results with regard to the 4 antigens used were as given in Table 35. Figures 13 and 14 represent the MIT results for test and control birds, respectively.

Table 35: PERCENT MACROPHAGE MIGRATION INHIBITION FOR S. GALLINARUM SOMATIC ANTIGENS IN EXPERIMENTALLY - VACCINATED BIRDS

Day Post-vaccination		% Macrophage migration inhibition			
		Ag1	Ag2	Ag3	Ag4
26	T	21	32	27	27
	C	NI	18	5	16
32	T	43	38	45	46
	C	45	39	70	53
39	T	15	15	NI	NI
	C	1	6	40	5
46	T	46	20	46	-
	C	35	28	47	-
I		2	-	16	-

Key

T means "test birds"
C means "control birds"
I means "clinically - infected birds from the field"
Ag1 means "S.Kiambu"
Ag2 means "S.Senftenberg"
Ag3 means "S.gallinarum" strain L25
Ag4 means "S.strasbourg"
NI means "no inhibition"
- means "not done"

FIGURE 13

MIT RESULTS FOR THE TEST BIRDS WITH REGARD TO THE 4 ANTIGENS USED

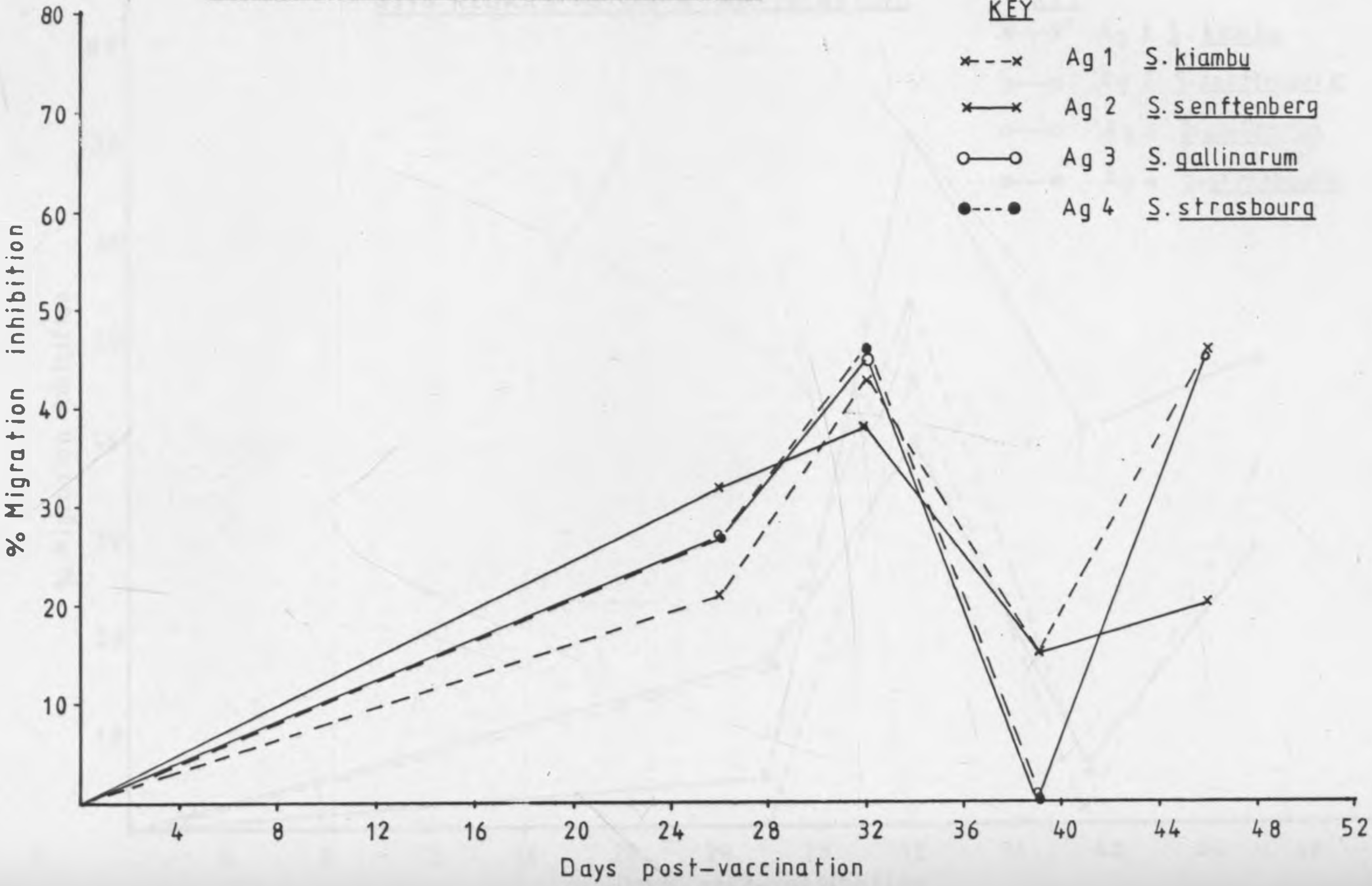
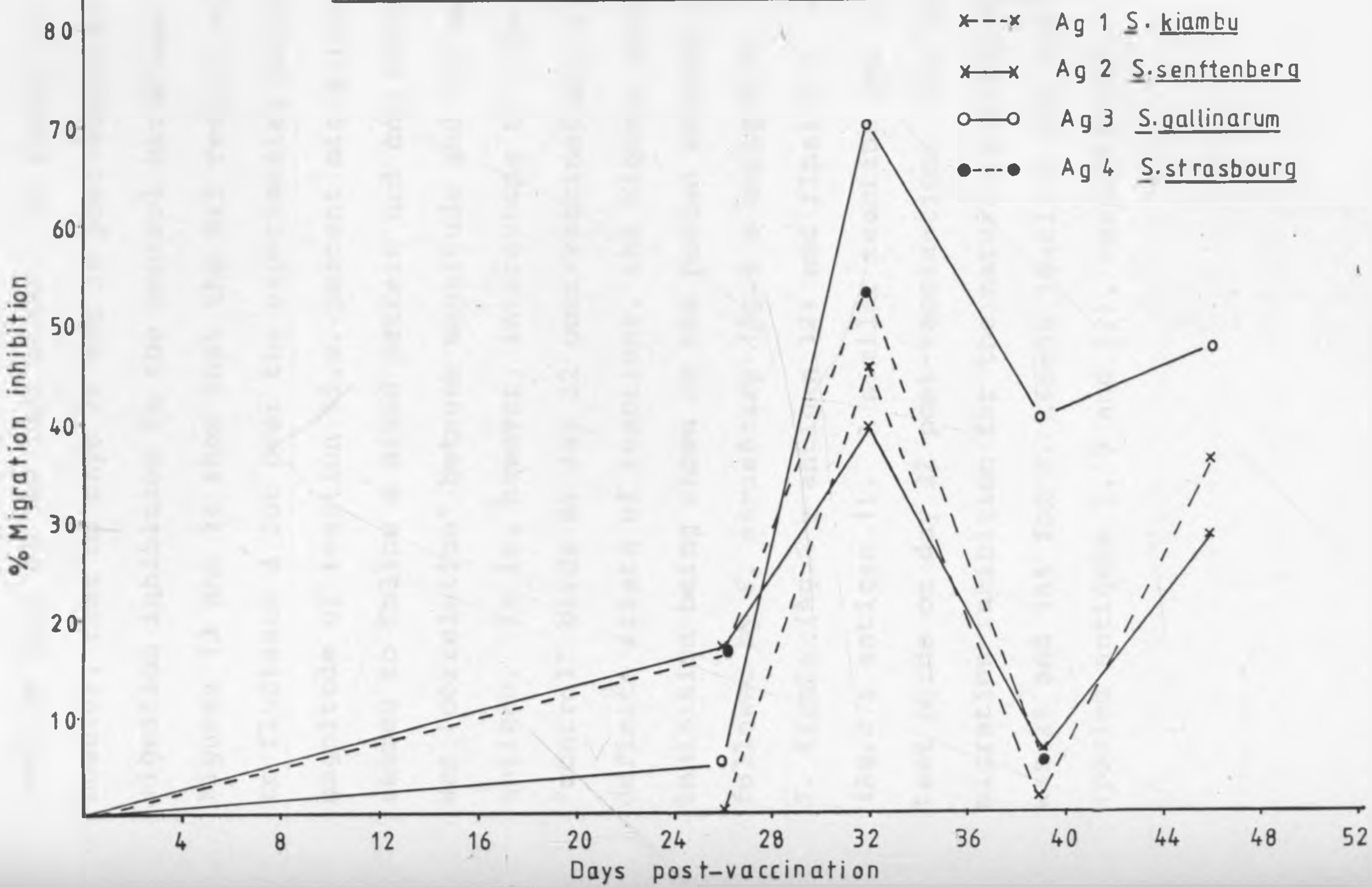


FIGURE 14

MIT RESULTS FOR THE CONTROL BIRDS WITH REGARD TO THE 4 ANTIGENS USED



As with the serological tests, control birds also showed macrophage migration inhibition, which in some cases, namely the 32nd and 46th day post-vaccination, was as good as that of the test birds. It should be noted, however, that on days 26 and 39 post-vaccination, the migration inhibition in the control birds was negligible. Figures 13 and 14 show that the MIT results also tended to fluctuate a lot over the experimental period. The magnitude of reaction (i.e. percent migration inhibition). seemed to follow a mixed pattern and one cannot say there was "correlation" between magnitude and any one *S. gallinarum* antigen. It is, however, interesting to note that in the "control" birds at day 32 post-vaccination, there was definite strata of reactions, the highest magnitude of inhibition being shown on the pooled antigen (Ag.3), followed by *S. strasbourg* (Ag.4 ≡ antigen 9), then *S. Kiambu* (Ag.1 ≡ antigen 12) and finally *S. senftenberg* (Ag.2 ≡ antigen 1). A similar reaction was shown in the test birds on day 32 post-vaccination. The percent migration inhibition for the naturally infected birds was 2% and 15% for *S. Kiambu* (antigen 12) and *S. gallinarum* (pooled antigens 1, 9 and 12), respectively.

5. DISCUSSION AND CONCLUSION

This study was designed to cover three aspects of the fowl typhoid disease in chickens, namely:- (i) the disease in general, which included pathogenesis and pathogenicity of selected *S. gallinarum* isolates; (ii) the immune response to the *S. gallinarum* bacterium; and (iii) the various ways of controlling the disease, including vaccination using both the currently-used vaccine strain, CN 180 and the newly - developed vaccine strain, L46. Various experiments were carried out and these gave an indication of the nature of the disease, as well as the disease-causing-organism, as it occurs in Kenya; the bird's immune responses to the *S. gallinarum* organisms; and the efficacy of the two vaccines. This information helped in explaining the occasional vaccination breakdowns that have been detected in the field.

The pathogenicity study done using day-old chicks, including LD_{50} calculations, indicated that various *S. gallinarum* isolates had different pathogenicities (LD_{50} values). This indicates a strain difference although one should note, here, that any reduced virulence may be due to differences in the maintenance of different isolates on artificial media, resulting in varying degrees of attenuation

(Pomeroy, 1972). However, the post-mortem lesions of the dead birds were classically the same as those reported in literature on fowl typhoid disease (Pomeroy, 1972). The livers of the dead birds yielded pure cultures of *S. gallinarum* and the average liver bacterial counts ranged between 10^7 to 10^8 organisms per gramme of tissue. This was similar to that found by Collins (1974) in mice infected with *S. enteritidis*. Although in some intracellular bacterial parasites, virulence *in vivo* is often correlated with the ability to survive and multiply in macrophages *in vitro*, this was shown not to be the case with *Salmonella* (Collins, 1971). He found that salmonellae, differing in virulence, were inactivated at similar rates intracellularly in cultures of mouse macrophages.

The adult birds infected orally with *S. gallinarum* did not show any signs of disease while those infected intraperitoneally died at a rate proportional to the dosage given. This finding is the same as that of other workers (Brownell *et.al.*, 1969; 1970; Leaney *et.al.*, 1978; Williams, 1972). In explanation of the difficulty to infect birds through the oral route, Abrams and Bishop (1966) suggest that the intestinal microbial flora offers resistance to infectio~~u~~ns both directly, through competing for limited nutrients (Freter, 1962) and/or through production of antibacterial substances (Bohnhoff *et.al.*, 1964; Meynell, 1963); and indirectly by moulding the structure of the lamina propria, the life-cycle of the mucosal cells and the mucosal surface area (Abrams *et.al.*, 1963; Gordon and Bruckner - Kardoss, 1961). The intestinal microbial flora was also shown to alter enteric infection by increasing intestinal emptying (Abrams and Bishop, 1966; Dixon, 1960). Abrams and Bishop (1966) showed that when intestinal emptying was prevented by ileal ligation before challenge of the mice with *S. typhimurium*, both the germ-free and conventional mice showed the same extent of intraluminal growth and translocation of the *S.typhimurium*. This supports the results of Miller and Bohnhoff (1962) who found that *S. enteritidis* is capable of multiplying in the small bowel of the mouse if peristaltic arrest is induced by morphine. Formal *et.al.* (1963), working on experimental models of shigellosis in guinea pigs, found that pharmacologically induced impairment of intestinal motility resulted in maintenance of sufficiently large numbers of pathogenic organisms within the small intestine for a long enough time that significant penetration of the mucosa occurred.

Williams (1972) mentioned that chronic intestinal carriers of paratyphoid infections are common, but that "disease" seldom occurs except in young fowl or in mature birds subjected to some stressing conditions. Factors recognised as "stressors" in poultry are listed by Von Faber (1964) as:- muscular fatigue, cold, heat, wetness, starvation, limitation of food and water, deprivation of water, surgical trauma, debeaking, anoxia, infections, crowding, and certain drugs, chemicals or antibiotics administered orally or parenterally. Bierer and Eleazor (1965) reported that one-week-old chicks naturally infected with *S. montevideo* developed clinical signs when deprived of feed and water for 3-5 days. Bierer *et.al.* (1966) later reported creating intestinal lesions by depriving uninfected chicks of feed and/or water, and suggested that such lesions might provide portals of entry for infections. Similar results were reported by Brownell *et.al.* (1969), who showed that stressing by withholding water creates an intestinal environment that is condusive to establishing an intestinal salmonella infection that will persist longer than in "normal" intestines. Seidman and Arnold (1932) demonstrated that *S. typhimurium*, administered orally, penetrated the intestinal wall and invaded the visceral organs much more readily in rats on a vitamin-A-deficient diet than in rats on a normal diet. Concurrent infections with, for example, *Escherichia coli* or coccidia have been shown to increase the susceptibility of chickens to salmonellosis (Brownell *et.al.*, 1969; Stephens *et.al.*, 1964). Reduction or neutralisation of the acidic contents of proventriculus and gizzard by administering an alkali, like magnesium carbonate or sodium bicarbonate, was also shown to increase the susceptibility of chickens to salmonella (Brownell *et.al.*, 1969; Smith, 1955). This phenomenon of "stressors" acting as predisposing factors to disease, indicates the possible role of poor husbandry in increasing the salmonella problem in flocks that may be exposed to infection from outside or by indigenous sources. The establishment of intestinal infection has been shown to be influenced by both bird age and numbers of organisms (Milner and Shaffer, 1952; Sadler *et.al.*, 1969). The incidence of clinical disease has been shown to decrease rapidly with age (Severens *et.al.*, 1944; Solomon, 1968a). Turnbull and Snoeyenbos (1974) had similar findings with respect to intestinal penetration and localisation of *S. enteritidis* in chicks of various ages.

Leaney *et.al.*, (1978) reports on the cloaca being the possible port of entry for salmonella infections in chickens. Experiments done using this route have yielded more predictable results in terms of mortality and numbers of organisms in the liver, spleen, and intestinal tissues, than when infected through the oral route. Based on quantitative data, the course of the cloacal infection appeared similar to that given by the oral route in hatchlings; however, the number of organisms required to establish infection by the former route was significantly less. The habits of birds living in Salmonella - contaminated environment of poultry sheds would permit small numbers of organisms to gain access by this route. Forsythe *et.al.*, (1967) have, however, shown that the hen has remarkable resistance to the occasional challenge with large numbers of *Salmonella* organisms, even when the challenge is directed at an organ exposed by surgery.

In this experiment, presence of *S. gallinarum* in the eggs, which is an indication of ovarian transmission, was not demonstrated.

The results on the pathogenesis experiment using 45-day-old groups of chickens, showed that intraperitoneally (IP)-inoculated birds had organisms in their livers and spleens as early as 3 hours post-inoculation while the orally-inoculated birds had organisms in their livers 24 hours post-inoculation. This was similar to the findings of Leaney *et.al.*, (1978) and Turnbull and Snoeyenbos (1974). The organisms were not detected in the caeca of I.P.-inoculated birds, and gizzard, duodenum and caecum of orally-inoculated birds. This was contrary to the findings of Leaney *et.al.*, (1978) and Turnbull and Snoeyenbos (1974) who detected salmonellae in the cloacal and cecal cultures of both intraperitoneally and orally-infected birds. The failure of detecting the presence of the organisms in the heartblood samples, except at 6 hours post-intraperitoneal-inoculation, suggests that bacteraemia was transient and that, in orally-inoculated birds, the organisms found their way to the internal organs through tissue penetration rather than through bacteraemia.

Salmonellae belong to the group of enteric pathogens which invade the intestinal epithelial barrier, multiply in the mucosa, and enter the circulation (Turnbull and Snoeyenbos, 1974). Since orally-introduced salmonellae exhibit a predilection for the caudal ileum and the ceca of chickens (Brownell *et.al.*, 1969; Fanelli *et.al.*, 1971), transit from the lumen into the intestinal tissues has been assumed to occur there. Turnbull and Richmond (1978), however, demonstrated that salmonellae had a potential ability to enter the mucosa at any level of the intestine. The incidence of penetration fell rapidly with age (Turnbull and Snoeyenbos, 1974).

When 15-week-old cockerels were vaccinated with *S. gallinarum* strains L46 and CN 180, and their immune response monitored by MIT and IHA over a period of 37 weeks, they showed a good immune response towards the two vaccines. The response, as measured by percent macrophage migration inhibition compared well between L46 and CN 180, although there were times when L46 gave higher values. The response fluctuated a lot as the experiment progressed and L46 seemed to be more effective than CN 180 in maintaining the response. Overall, the L46-vaccinated birds showed higher IHA titres than the CN 180 - vaccinated birds. The titres fluctuated a lot throughout the experimental period. The CN 180 - vaccinated birds showed a more gradual increase in titre and there were less fluctuations than in L46-vaccinated birds. The IHA results, thus, matched well with the MIT results, where L46-vaccinated birds, in general, gave higher immune response than the CN 180 - vaccinated birds. This shows that the isolate L46 induces a more intense immunological reaction than CN 180.

flactuation

The flucation of titres in serological tests has been reported by other workers (Gordon and Garside, 1944; Gordon and Buxton, 1945; Karlshoj and Szabo, 1949; Blaxland and Blowers, 1951). The positive responses to MIT and IHA in control birds is not surprising. Although this may mean the birds had been exposed to the organism or to a cross-reacting antigen, titres to IHA have been detected in animals known not to be exposed to the respective antigen (Wray *et.al.*, 1975; Magwood and Bigland, 1962). False positives with regard to MIT have also been reported (Timms and Alexander, 1977; Timms and Cullen, 1976, Rocklin, 1976).

The experiment based on immunising one-day-old chicks showed that there was very little protection rendered by the vaccine, CN 180, regardless of the dosage given. This correlates with the observation of Wolfe and Dilks (1948) who found that newly-hatched chicks failed to respond or responded very weakly to a parenteral injection of bovine serum. Wolfe *et.al.*, (1957) showed that immunoglobulins demonstrable by serological test procedures in avian species are stimulated more readily and reach higher levels as birds mature. They found that serological maturity was reached between 22 and 25 weeks. Hirata and Schechtman (1960) reported that chickens do not attain full capacity for antibody formation until they are about 22 weeks old. However, Williams and Whittemore (1975) found that birds 6 weeks old and older responded well with high antibody titres when infected orally with *S. typhimurium*. Gordon *et.al.*, (1959) found that fowl·typhoid live-attenuated vaccine gave better immunity when used at 8 weeks of age than at 4 weeks. Kodama *et.al.*, (1976), working on age-dependent resistance of chickens to *Salmonella in vitro*, using splenic phagocytes, found that bactericidal activity against *S. pullorum* increased with age, but the activity of the splenic phagocytes was the same, regardless of age.

Fifteen-week-old-pullets were vaccinated with CN 180 and L46 vaccines and challenged with virulent *S. gallinarum* isolate L46 at intervals of 4 weeks, 8 weeks, 13 weeks and 17 weeks post-vaccination. Birds were sacrificed, at various intervals post-challenge, and liver colonisation by bacteria, liver/spleen hypertrophy indices and immune response, both humoral (IHA) and cellular (MIT) were assayed. The results showed that both CN 180 - and L46 - vaccinated birds followed a similar immune reaction when monitored by MIT. In general, the IHA titres for L46-vaccinated birds were higher than those for CN 180 - vaccinated birds. This could be due to the effect of homologous challenge, since virulent L46 was used as the challenge organism (Davies and Kotlarski, 1976; Collins, 1968). The rates of liver colonisation by *S. gallinarum* organism in both CN 180 - and L46-vaccinated birds were more-or-less the same, although in the 4th challenge, the L46-vaccinated birds showed much more resistance to bacterial multiplication than the CN 180 - vaccinated birds.

The MIT results were highest in birds used for the second challenge, the degree of inhibition reducing in the 3rd and 4th challenges. This pattern was similar in both CN 180 - and L46-vaccinated birds. This observation correlates with that of resistance to liver colonisation by *S. gallinarum* as shown in Tables 18 and 19, respectively, where livers loaded with large numbers of bacteria, i.e. 2,200 organisms per gramme (org/g) in CN 180 - vaccinated birds on day 1 post-2nd challenge and 25,000 org/g in L46-vaccinated birds on day 2 post-2nd challenge, were brought to zero on the next sampling i.e. day 2 post-2nd challenge in CN 180-vaccinated birds and day 3 post-2nd challenge in L46-vaccinated birds, thereafter, the numbers were controlled at below 400 org/g. Although the migration inhibition values for birds used in the 3rd and 4th challenges were lower than in those used for the 2nd challenge in both CN 180 - and L46-vaccinated birds, the birds used for the 3rd and 4th challenges seemed to have controlled the numbers of bacteria in their livers much more effectively than those used for the 2nd challenge, i.e. they maintained the numbers of bacteria at not more than 600 org/g. The only exception, here, was CN 180, 4th challenge, where the number shot to 410,000 org/g on day 6 post-challenge, but this was brought down to 100 org/g and later to zero on days 10 and 12 post-challenge, respectively. These observations, thus, indicate that the degree of macrophage migration inhibition, which is an *in vitro* correlate of delayed hypersensitivity reaction (Timms, 1979; Timms and Alexander, 1977; Timms and Cullen, 1976; Rocklin, 1976), is not directly proportional to liver bacterial clearance rate, in this case. The close correlation between reactions of L46 - and CN 180 - vaccinated birds has been exhibited both in liver colonisation and MIT results, thus, indicating that the two vaccines induce similar immune responses in birds. Statistical analysis showed that there was no significant difference between liver/spleen hypertrophy and resistance to bacterial multiplication as indicated by bacterial localisation in the liver. The results showed that actual recovery of bacteria from respective livers gave better indication of infection than liver or spleen hypertrophies.

This method of detecting challenge populations in the vaccinated and control animals has been considered a superior parameter to the method of recording death or survival of the host (Collins, 1971; 1972a; 1974; Mackaness *et.al.*, 1966). Death may be due to toxaemia as for *S. typhosa* in laboratory animals, rather than the infection (Diena *et.al.*, 1973). The development of a protective acquired resistance can only be inferred from the presence of a significant shift in the systemic bacterial growth rate at an earlier stage of the challenge infection. Such an accelerated antibacterial response must be sufficient to prevent completely the development of clinical attack of the disease (Blanden *et.al.*, 1966; Collins, 1969b), a criterion widely used in field trials of human typhoid vaccines (Cvjetanovic and Uemura, 1965; Spaun and Uemura, 1964). The effective vaccine will so sensitise the host that an accelerated recall of the earlier induced cell-mediated immunity will occur almost immediately following the re-exposure to the parasite. The time required to regenerate this type of one of the most progressive antibacterial responses is perhaps one of the most important parameters of the immune reaction (Collins, 1968). To get the complete picture of the immune response, however, one should combine the death or survival data, the assessment of the bacterial growth rate *in vivo* and the demonstration of a cell-mediated type of immune response (Collins, 1974).

The challenged control birds gave varying IHA titres ranging from 64 to 128, over the 6 experimental days, post-challenge. This may be due to a previous exposure of the birds to the organism or to a cross-reacting antigen or due to non-specific reactions as recorded by other workers (Wray *et.al.*, 1975; Magwood and Bigland, 1962). The MIT response, in these birds, has shown a gradual increase. The birds sacrificed 1 day and 2 days post-challenge showed no macrophage migration inhibition. One of the two birds sacrificed on the 3rd day post-challenge had a percent inhibition of 29.7. The two birds sacrificed on the 6th day post-challenge had percent inhibitions of 49 and 59.4, respectively. This is expected since cell-mediated-immunity has been shown to develop as early as 3rd day post-vaccination (Timms and Alexander, 1977; Timms and Cullen, 1976; Timms and Bracewell, 1981).

Antibiotics are being used extensively in the treatment of fowl typhoid (Pomeroy, 1972; Snoeyenbos, 1972; Poultry Clinic, University of Nairobi, unpublished data; Muravev, 1978). The surveys done on the antibiotic sensitivities of the various *S. gallinarum* isolates showed that antibiotics like furazolidone, neomycin, chloramphenicol, tetracycline, penbritin (ampicillin), were very effective on a number of the isolates. However, the results indicated the presence of resistance to one or more antibiotics, more so to streptomycin and sulphonamides. A few showed resistance to Ampicillin. This drug resistance, single or multiple, has been reported in enterobacteria by a number of workers (WHO report, 1980; Davies *et.al.*, 1978; Sang *et.al.*, 1985; Kim *et.al.*, 1980; Smith *et.al.*, 1981). The presence of antibiotic resistance in any enterobacterium pauses a problem in treating fowl typhoid in chickens, since the resistance factor can be transferred from one bacterium to another through conjugation (Watanabe, 1963; Anderson, 1968; Shrago *et.al.*, 1986). Indeed, Biru *et.al.*, (1981) and Dezfulian and Naghashfar (1981) have actually demonstrated the transfer in enterobacteria.

For cleaning the chicken houses and other surroundings, various disinfectants can be used (Pomeroy, 1972; Snoeyenbos, 1972). These include acids, alkalis, salts, heavy metals, halogens, formaldehyde, ethylene oxide, cationic detergents like quaternary ammonium compounds, and phenols (Davis *et.al.*, 1973; Hugo, 1970). This thesis reports on disinfectant sensitivities done on 6 disinfectants, namely:- lysol, pynol, kerol, biodan, bromosept and municipal fluid.

Bromosept was found to be the most efficient disinfectant, followed by lysol, then kerol, then biodan and pynol and lastly municipal fluid. Apart from bromosept and lysol, the disinfectants were shown to be ineffective at the recommended concentrations; higher concentrations were needed for any significant effect. The municipal fluid had no effect at all the dilutions studied. There was only a narrow zone of inhibition (11-12mm diameter) effected by the neat chemical. Considering the control (type) cultures surveyed, *E.coli* gave more-or-less the same reactions as the *S. gallinarum* isolates. *Staph. aureus* gave similar reactions as the *S. gallinarum* isolates when tested with lysol, biodan and bromosept, but showed greater sensitivity to pynol and kerol using both the recommended concentrations and higher ones. Bizimenyera (1986) carrying out a similar study, covering both gram-positive and gram-negative bacteria, found that kerol and pynol gave better effect on gram-positive than on gram-negative bacteria.

Disinfection, as opposed to sterilisation, does not render a surface completely free from all viable microorganisms (Davis *et.al.*, 1973). It, however, renders it free from infection (Davis *et.al.*, 1973; Hugo, 1970).

A disinfectant is usually a chemical agent which destroys disease germs and other harmful microorganisms but not the bacterial spores (Hugo, 1970), and it is used on inanimate objects only (Patterson, 1932). Of the various disinfectants in the market, alkylating agents, like formalin (or formaldehyde), have been reported as being most effective disinfectants (Davis *et.al.*, 1973). These alkylating agents, in contrast to other disinfectants, are nearly as active against spores as against vegetative bacterial cells, presumably because they can penetrate easily (being small and uncharged) and do not require water for their action (Davis *et.al.*, 1973). Williams (1980) reports on destruction of salmonellae in poultry litter using formalin.

Vaccination is the third method of controlling fowl typhoid. Dead vaccines have been shown to be of little value in the control of salmonellosis (Winmill, 1961; Smith, 1956b; Collins and Carter, 1972; Collins, 1969a,b; 1971;1974; Diena *et.al.*, 1977; McNutt, 1926). This is because the killed vaccines give rise to humoral immunity only and not to cell-mediated immunity (Davies and Kotlarski, 1976; Solotorovsky and Soderberg, 1972). As organisms that can survive within normal macrophages without being degraded (intracellular organisms) (Collins, 1974; WHO Scientific group, 1973; North, 1974), protection against them has been shown to be through delayed hypersensitivity reaction which is cellular in nature (Collins, 1971; Mackaness, 1967). However, antibodies have been shown to play some role in the protection against salmonellosis (Cameron, 1976; Davies and Kotlarski, 1976; Collins, 1968). Solomon (1968b) has reported that circulating antibodies, whether agglutinating, incomplete, cytophilic or bactericidal, do not appear to be protective in avian *Salmonella* infections. Maternal antibodies of both IgG and IgM classes are transmissible to embryos (Buxton, 1952; Kramer, 1973) but no evidence was seen that these afford protection. The present study, working on attenuated smooth vaccines L46 and CN 180, has shown that both of these give good protection as assayed by detection of cell-mediated immunity and also by assessment of the bacterial growth rate *in vivo* through doing bacterial liver counts after challenging the vaccinated birds with a virulent strain of *S. gallinarum*.

The reported success in controlling *Salmonella* infections by feeding chicks with anaerobically - cultured caecal/intestinal or faecal contents (mixed broth culture) of adult fowls (Shneitz *et.al.*, 1981; Snoeyenbos *et.al.*, 1978; Seuna *et.al.*, 1978; Rigby and Pettit, 1980) is mainly due to competitive exclusion of salmonellae by the heavy flora that develops in the gut (Shneitz *et.al.*; 1981; Snoeyenbos *et.al.*, 1978). It is also due to production of antibacterial substances by resident microflora; and by slowing down of intestinal motility (Abrams and Bishop, 1966).

Although mention has been made to the various ways of controlling the disease, it should be noted that birds exposed to salmonellae, even if treated, remain carriers of the organisms (Pomeroy, 1972; Collins, 1974; Williams, 1972). These serve as a constant source of disease organisms which can infect other susceptible birds. The only final solution to this problem, as practised in a number of countries, is blood-testing and eradication of all positive reactors to fowl typhoid/pullorum disease (Pomeroy, 1972; Schwartz, 1972). The blood-testing is done using RWBPT and all positive reactors or the whole flock is killed and the birds buried or burnt. The houses are then thoroughly disinfected, preferably followed by fumigation with formalin, before any other birds are brought in (Blaxland *et.al.*, 1958; Robinson, 1970; Snoeyenbos, 1972; Schwartz, 1972). The RWBPT has been shown not to detect all carriers (Karlshoj and Szabo, 1949; Magwood and Bigland, 1962; Smyser *et.al.*, 1966). Environmental sampling and culturing (litter and droppings) may serve as one of the diagnostic tools for detecting flock infections (Baker *et.al.*, 1966; Smyser *et.al.*, 1966; Snoeyenbos *et.al.*, 1967). Other serological tests like tube agglutination test (Jones, 1913), microagglutination and microantiglobulin tests (Williams and Whittemore, 1971; 1972), indirect haemagglutination test (Neter *et.al.*, 1952a) and fluorescent antibody methods (Georgala and Boothroyd, 1954; Haglund *et.al.*, 1964) may also be used. Cloacal swabbing and culture was found inadequate in detecting salmonella infections (Williams and Whittemore, 1976b); Bebora *et.al.*, 1979).

Where eradication cannot be practiced, hygiene must be practiced to the maximum, to make sure the disease is not transmitted to other susceptible birds. Hatchery and flock sanitation is most important in prevention and control of the disease. Adult intestinal carriers are the main source of infection in poultry.

The results have indicated that the isolates recovered and characterised by the various methods were different strains. The difference is either in their biochemical processes as indicated by their reaction to the antibiotics and disinfectants sensitivity testing (Appendices 7 and 8); or in their phage receptors, as indicated in the phage-typing experiment where they showed differing areas of lysis (Table 31). Care was taken to seed more-or-less the same number of organisms on the plates, so as to eliminate the effect of varying bacterial concentrations on the tests done. Phage-typing has been used as a means of distinguishing between strains of the same serotype (Lilleengen 1947; 1948; 1952; Craigie and Yen, 1938) and has shown a great value in epidemiologic and epizootiologic investigations of salmonella outbreaks as well as other diseases (Barker and Tyc, 1982).

From the results got, it was not possible to pin-point a "marker" for any one of the *S. gallinarum* isolates, since there was none that behaved *distinctly* different from the others. However, the following isolates were almost distinct from others in various aspects:-
(i) isolate L61 showed high sensitivities to both streptomycin concentrations: 10µg and 25µg (Appendix 7), while the other isolates showed much lower or no sensitivity; ii) considering reactions to Bromosept^{R}, isolate L49 gave exceptionally larger inhibition zone-diameters than the other isolates for all the concentrations used (Appendix 8); and (iii) isolate L15 was not attacked by phages 2 and 5 (Table 31), while the other isolates showed some sensitivity to all the phages. More work needs to be

done to prove that these properties are permanent before one can take them as distinctive behaviours. Isolates L46 and CN 180 gave reactions that were more-or-less the same as the rest of the isolates.

The study of the relationship between the immune status of an infected bird (IHA titre and/or MIT) and any one of the three *S. gallinarum* somatic antigens was prompted by the fact that the nature of the immunogen in cases of *Salmonella* vaccines has not yet been clearly demonstrated (Misfeld and Johnson, 1978; Cameron, 1976; Killion and Morrison, 1986). In this study, it was observed that the 3 antigens gave rise to varying degrees of immunity as shown by IHA titres and degrees of macrophage inhibition (M1), but there was no constant pattern relating higher IHA titres or higher degrees of M1 to one particular antigen. This suggests that the *Salmonella* 'O' antigens may not be the antigens giving rise to protective immunity. This point is supported by Misfeld and Johnson (1978), Collins (1974), Smith (1956b), Cameron (1976) and Killion and Morrison (1986). The fact that rough mutants of salmonellae, which are denuded of much of their cell-wall polysaccharide ('O' antigens) will induce immunity (Smith, 1956b ; Arda, 1971; Gupta and Mallick, 1976a,b; Silva *et.al.*, 1981; Germanier, 1970) indicates that the 'O' antigens do not play an important role in this respect. Furthermore, the observation that even closely related smooth strains of *Salmonella* may differ enormously in their virulence for mice (Collins *et.al.*, 1966; Furness and Rowley, 1956) and for chicken (Karthigasu and Jenkin, 1963; Smith, 1956b; Solomon 1968a); and also the failure to protect between heterologous strains (Davies and Kotlarski, 1976; Collins, 1968), despite close similarities in the lipopolysaccharide content of the organisms (Collins, 1967) shows that the specificity involved in the cellular mechanism of defence may be independent of the 'O' somatic antigens. Indeed the *S. gallinarum* 9R (rough variant) vaccine was more effective than the *S. enteritidis* E.20 live smooth vaccine in clearing the challenge organisms, *S. gallinarum*, from the chicken's body (Padmanaban and Mittal, 1981). These two organisms have the same somatic antigenic formula of 1,9,12 (Kauffmann, 1975).

Early work by Venneman and co-workers (Venneman and Bigley, 1969; Venneman, Bigley and Berry, 1970) described the immunogen as a stable RNA molecule that did not require an adjuvant to be effective. Further studies of Venneman (Venneman, 1972) indicated the molecule to be either RNA or an undefined polysaccharide of greater than $0.3\text{x}10^6$ daltons. Johnson presented contrasting evidence showing that purified *Salmonella* ribosomal protein, free from contamination with RNA, could protect mice against challenge with virulent organisms (Johnson, 1972; 1973). Other authors have reported the same (Barber *et.al.*, 1972, cited by Cameron C.M., 1976). Success in usage of ribonucleic acid-protein fractions as protective vaccines has been reported (Smith and Bigley, 1972a; Misfeld and Johnson, 1978; Pepper *et.al.*, 1976; Waiyaki, 1974; Eisenstein and Angerman, 1978). The findings of Smith and Bigley (1972a) suggested that both RNA and protein may be necessary for optimum immunogenicity. Their investigations showed that a protein-rich fraction (NP), obtained from ethanol-precipitated ribosomes containing 1% RNA contamination, gave less protection than their NP fraction containing 10% RNA contamination (Smith and Bigley, 1972a). These results could also be duplicated if the NP was mixed with the synthetic polynucleotide polyadenylic acid: polyuridylic acid (Smith and Bigley, 1972a). The evidence suggests that protein or some contaminant may be the immunogen and that RNA may be serving as an adjuvant. A case where immunisation has been achieved in experimental mice using other membrane proteins has been reported (Kuusi *et.al.*, 1979; Svenson *et.al.*, 1979; Killion and Morrison, 1986). The presence of a component with toxic properties similar to those of endotoxin has, however, been found present in active vaccines but not in weak ones. This was shown by the ability of effective vaccines to kill lead acetate-sensitised mice and to induce tolerance to endotoxin (Hoops *et.al.*, 1976). Winmill (1961) showed that the 1909 S (smooth variant) attenuated *S. gallinarum* vaccine gave more satisfactory protection to chickens vaccinated at various ages than the 1909 R (rough) variant) *S. gallinarum* vaccine. The birds vaccinated with the smooth variant survived a challenge which killed 95% of the unvaccinated controls while only 20% of the birds vaccinated with the rough variant were protected. Smith (1956b) reported similar findings. A case where antibodies to *Salmonella* polysaccharide antigens have shown protection against challenge with virulent *Salmonella typhimurium* has been reported (Colwell *et.al.*, 1984).

From the present experimental birds, it was observed that sera/cells from the same group of birds showed stronger reactions with serology than with macrophage migration inhibition test. To explain this, reference is given to the fact that there was a group of vaccinated cockerels, and that only 3 of these, randomly selected, were bled, at any one time, for the two tests. The sera/cells from the 3 birds were pooled and worked on together. In both cases there was, therefore, an element of dilution depending on the original concentrations of the respective factors in the individual birds. The antibodies may have been in very high titres so that, even though diluted, the resultant titre was still high enough to cause strong agglutinations with stained *S. pullorum* antigen, as well as give high titres with TAT and IHA. This may not have been the case with the T-lymphocytes. There may have been few sensitised T-lymphocytes, hence easily diluted and left with very few; and since the test is run only for a few hours, the time may not have been sufficient for them to multiply enough to cause any distinct effect. This is explained better when 4 known infected birds (indicated as I in Tables 33 and 35) were bled and their sera/cells pooled. One of these birds had shown negative RWBPT reaction while the other 3 were strongly positive. Since the cells were pooled, and there was dilution of the sensitised lymphocytes, the result was a very low percent migration inhibition (2% and 16%). However, TAT titres ranged from 320 to 640 while IHA titre was 1,024. This suggests that it is better to deal with individual specimens (chickens) and take average results later, rather than pooling the sera/cells before doing the tests.

Overall, the TAT detected higher titres to *S. gallinarum* (pooled antigen) than the IHA (Table 34), although the peak titre for IHA was 512 while that for TAT was 320. This was contrary to other workers' findings that IHA is more sensitive than TAT (Wray *et.al.*, 1975; Neter *et.al.*, 1954; Smith *et.al.*, 1972; Carrere and Roux, 1952). The detection of titres as high as 320 with TAT and 256 with IHA in control birds, indicates that these birds may have been exposed to the organisms or to some cross-reacting antigen. However, titres as high as 20 in known *S. virchow*-negative birds (Smith *et.al.*, 1972) and as high as 80 in known *Salmonella* - negative cattle (Hall *et.al.*, 1978; Hinton, 1973; Field, 1948; and Clarenberg and Vink, 1949) have been reported with regard to TAT; and Wray *et.al.*, (1975) have reported detecting very high IHA titres in cattle with no history of *S. dublin* infection. Magwood and Bigland (1962) have also reported on the non-specific reactions observed in haemagglutination tests of sera from non-infected birds. In some cattle which were positive for *S. dublin* on culture, neither TAT nor IHA tests detected diagnostic levels of antibodies (Wray *et.al.*, 1975). Magwood and Bigland (1962) reported the same in turkeys. MIT also gave positive results in some control birds although, in some cases, the percent migration was negligible. This has been mentioned by other workers (Timms and Alexander, 1977; Timms and Cullen, 1976; Rocklin, 1976). Rocklin (1976) relates this to various factors, namely:- antigen toxicity, alkaline pH, serum factors or some antibody-antigen complexes. The effect of endotoxin on MIT has been documented (Alexander and Evans, 1971; Cohn and Morse, 1960; Fox and Kausalya, 1980; Landy and Braun, 1964; Morrison and Ryan, 1979). A case of a vaccinated bird that failed to give positive result has been reported (Timms and Alexander, 1977).

The snag behind this exercise was that one could not rule out the possibility of cross-reactions as a result of the other antigens screened here. For example, when using *S. strasbourg* to screen for antigen 9 of *S. gallinarum*, any agglutination detected may have been due to either the antigen 9 or antigen 46, since the somatic antigenic formula of *S. strasbourg* is 9,46 (Kauffmann, 1975). It would have been necessary to screen the same serum with another organism that had antigen 46 but not antigen 9, to rule out the possibility of false positives. Unfortunately, this was not possible because antigen 46 is found in *Salmonella* group D_2 only and all serotypes in this group have the somatic formula of 9,46 - they only differ in their flagella antigens (Kauffmann, 1975). Similarly, one could not outscreen the possibility of the other antigens in *S. senftenberg* and *S. kiambu* causing cross-agglutinations because: (i) all strains that have antigen 4 have antigen 12 also (Kauffmann, 1975), hence cannot screen for antigen 4 in *S. kiambu* and (ii) all strains of group E_4 have antigens 1,3,19. One can screen for antigen 3 of *S. senftenberg* using members of groups E_1, E_2 and E_3, but antigen 19 is a characteristic of members of group E_4 only, hence cannot screen for it (Kauffmann, 1975).

In conclusion, the establishment of the existence of different strains of *S. gallinarum* both biochemically and serologically pauses a problem in controlling the disease, since various strains may behave differently to various antibiotics and disinfectants, let alone the birds' response to vaccination. This study has shown that both L46 and CN 180 can be used as the vaccine strains. Both give good immune responses. L46 has now been passaged on MacConkey agar and has shown attenuation. It cannot, however, be passed as a good vaccine yet. More work needs to be done to ascertain the stability of the non-pathogenic nature and the easiness of its reversion to virulent state. This organism showed an LD_{50} value of one (1) organism to day-old-chicks and, if there is reversion to virulence, it may cause more harm to the chicken industry than good. Field trials also need to be organised to show that what has been detected in the laboratory occurs in the field, and that the new vaccine can survive the mixed infections that occur in the field.

The vaccination breakdowns experienced in the field may have been as a result of possible emergence of new virulent strains of *S. gallinarum*. However, controlled experimental studies on typhoid fever, using human volunteers, have shown that the incidence of disease and apparent level of protection against a standardised oral dose of virulent *S. typhi* varies with the size of the challenge inoculum (Hornick and Woodward, 1967). An inoculum of more than 10^7 viable organisms will overwhelm any immunity induced by previous exposure to killed typhoid vaccine. This is consistent with the clinical finding that typhoid fever may occur in recently convalescent individuals (Marmion *et.al.*, 1953; Dupont *et.al.*, 1971b). It was also demonstrated that mice vaccinated with a living attenuated vaccine can still be superinfected (Collins, 1972b). Thus, even during convalescence, resistance to reinfection may decline so rapidly that second (although usually milder) attacks of the disease can occur if the infectious dose is large enough (Collins, 1972b) or the virulence of the reinfecting strain is very high (Marmion *et.al.*, 1953). *Salmonella* carriers have a high degree of resistance to reinfection and this state usually lasts as long as the primary infection persists *in vivo* (Collins, 1968; Hobson, 1957a,b). This echoes Collins' (1971) conclusion that antityphoid immunity is probably never absolute, even under ideal conditions, except, perhaps in the case of the permanent typhoid carrier. The same may be the case with fowl typhoid infection in chickens. This is in keeping with Reitman's (1967) statement that the ideal vaccine is one that retains the protective antigens of the bacterial cell, some of which might be extremely labile even to the extent of being non-demonstrable by present means of testing.

In this country, the CN 180 vaccine is administered intramuscularly at a dose of 10^3 organisms per bird. Administration is done once only at 8 weeks of age. This is in accordance to the observation that good immunity is established in birds from 8 weeks of age and older (Winmill, 1961; Gordon *et.al.*, 1959). The single-dose-administration is practised so as to ensure low antibody titres at laying time so as not to interfere with the fowl typhoid serological screening tests which may be done before start of lay. It may, therefore, be advisable to try and use a rough-strain-vaccine, since this has been shown not to produce agglutinating titres (Smith, 1956b). It has been shown to give good immunity in poultry (Smith, 1956b; Arda, 1971; Gupta and Mallick, 1976a,b; Silva *et.al.*, 1981). With this, booster vaccinations may be done without any interference with the screening tests.

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APPENDIX 1 - DEATH-RATE OF DAY-OLD CHICKS INFECTED WITH VARIOUS ISOLATES OF S. GALLINARUM AT VARYING CONCENTRATIONS:

CHICK DEATH-RECORD DAY-WISE

L56

	Concentration	$3.1x10^8$	$3.1x10^7$	$3.1x10^6$	$3.1x10^5$	$3.1x10^4$	$3.1x10^3$	$3.1x10^2$	$3.1x10^1$	$3.1x10^0$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	4	2	0	0	0	0	0	0	0
	Day 3	1	3	3	1	2	1	1	0	0
	Day 4	=	=	2	2	3	2	0	0	0
	Day 5	=	=	=	2	=	1	0	0	0
	Day 6	=	=	=	=	=	0	3	2	3
	Day 7	=	=	=	=	=	0	1	1	0

L21

	Concentration	$2.7x10^8$	$2.7x10^7$	$2.7x10^6$	$2.7x10^5$	$2.7x10^4$	$2.7x10^3$	$2.7x10^2$	$2.7x10^1$	$2.7x10^0$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	3	1	2	1	0	0	0	0	0
	Day 3	2	3	3	2	1	0	0	0	0
	Day 4	=	0	=	1	2	3	1	0	0
	Day 5	=	0	=	1	0	1	1	0	0
	Day 6	=	0	=	=	2	1	1	2	0
	Day 7	=	0	=	=	=	=	0	1	0

L32

	Concentration	$3.2x10^8$	$3.2x10^7$	$3.2x10^6$	$3.2x10^5$	$3.2x10^4$	$3.2x10^3$	$3.2x10^2$	$3.2x10^1$	$3.2x10^0$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	4	2	0	1	0	1	0	0	0
	Day 3	1	2	2	3	1	0	0	0	0
	Day 4	=	1	3	1	2	1	0	0	0
	Day 5	=	=	=	=	1	2	2	4	0
	Day 6	=	=	=	=	0	1	3	0	1
	Day 7	=	=	=	=	0	=	=	0	0

(Appendix I cont...)

L31

	Concentration	4.1×10^{8}	4.1×10^{7}	4.1×10^{6}	4.1×10^{5}	4.1×10^{4}	4.1×10^{3}	4.1×10^{2}	4.1×10^{1}	4.1×10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	5	3	1	0	0	0	0	0	0
	Day 3	=	2	0	4	0	1	0	0	1
	Day 4	=	=	3	1	4	0	0	0	0
	Day 5	=	=	1	=	1	4	3	3	1
	Day 6	=	=	=	=	=	=	0	0	3
	Day 7	=	=	=	=	=	=	2	0	=

L41

	Concentration	2.66×10^{7}	2.66×10^{6}	2.66×10^{5}	2.66×10^{4}	2.66×10^{3}	2.66×10^{2}	2.66×10^{1}	2.66×10^{0}	0.266
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	1	0	0	0	0	0	0	0	0
	Day 3	3	0	2	0	0	0	0	0	0
	Day 4	1	5	3	2	2	0	0	0	2
	Day 5	=	=	=	3	2	0	1	5	2
	Day 6	=	=	=	=	1	3	1	=	1
	Day 7	=	=	=	=	=	1	2	=	=

L55

	Concentration	2.66×10^{7}	2.66×10^{6}	2.66×10^{5}	2.66×10^{4}	2.66×10^{3}	2.66×10^{2}	2.66×10^{1}	2.66×10^{0}	0.266
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	0	0	0	0	0	0	0	0	0
	Day 3	4	0	0	0	0	0	0	0	0
	Day 4	1	4	2	4	0	0	0	0	0
	Day 5	=	1	2	1	2	1	0	0	0
	Day 6	=	=	1	=	3	1	2	0	0
	Day 7	=	=	=	=	=	1	0	0	0

(Appendix 1 cont...)

L7

	Concentration	3.6×10^8	3.6×10^7	3.6×10^6	3.6×10^5	3.6×10^4	3.6×10^3	3.6×10^2	3.6×10^1	3.6×10^0
No. dead	Day 1	1	0	0	0	0	0	0	0	0
	Day 2	3	0	0	0	2	1	0	0	0
	Day 3	1	3	1	1	0	0	0	0	0
	Day 4	=	1	2	0	1	3	0	0	0
	Day 5	=	1	0	1	0	0	1	1	0
	Day 6	=	=	1	1	1	1	1	0	0
	Day 7	=	=	0	0	0	=	1	0	0

L26

	Concentration	4.0×10^7	4.0×10^6	4.0×10^5	4.0×10^4	4.0×10^3	4.0×10^2	4.0×10^1	4.0×10^0	0.4
No. dead	Day 1	1	0	1	0	0	0	0	0	0
	Day 2	0	0	0	0	0	0	0	0	0
	Day 3	1	1	1	0	0	0	0	0	0
	Day 4	1	4	2	2	0	0	0	0	0
	Day 5	1	=	0	1	2	0	0	0	0
	Day 6	0	=	1	1	1	0	0	0	0
	Day 7	0	=	=	1	0	1	0	0	0

L23

	Concentration	4.0×10^7	4.0×10^6	4.0×10^5	4.0×10^4	4.0×10^3	4.0×10^2	4.0×10^1	4.0×10^0	0.4
No. dead	Day 1	1	0	1	0	1	1	0	1	0
	Day 2	0	0	0	0	0	0	0	0	0
	Day 3	0	1	0	0	0	0	0	0	0
	Day 4	3	1	0	0	0	0	0	0	3
	Day 5	0	2	1	0	1	0	0	0	1
	Day 6	1	1	0	0	0	0	0	0	0
	Day 7	=	=	0	0	0	0	0	0	0

(Appendix 1 cont...)

L47

	Concentration	2.7×10^{8}	2.7×10^{7}	2.7×10^{6}	2.7×10^{5}	2.7×10^{4}	2.7×10^{3}	2.7×10^{2}	2.7×10^{1}	2.7×10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	4	2	0	1	0	0	0	0	0
	Day 3	1	3	2	2	1	1	0	0	0
	Day 4	=	=	2	2	3	3	2	1	0
	Day 5	=	=	1	=	0	1	2	3	0
	Day 6	=	=	=	=	1	=	1	1	1
	Day 7	=	=	=	=	=	=	=	=	0

L46

	Concentration	2.53×10^{8}	2.53×10^{7}	2.53×10^{6}	2.53×10^{5}	2.53×10^{4}	2.53×10^{3}	2.53×10^{2}	2.53×10^{1}	2.53×10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	1	3	3	0	0	0	0	0	0
	Day 3	4	2	1	1	0	0	0	1	3
	Day 4	=	=	0	2	3	0	0	0	2
	Day 5	=	=	0	2	2	2	0	3	=
	Day 6	=	=	0	=	=	3	5	0	=
	Day 7	=	=	0	=	=	=	=	0	=

L3

	Concentration	4.0×10^{8}	4.0×10^{7}	4.0×10^{6}	4.0×10^{5}	4.0×10^{4}	4.0×10^{3}	4.0×10^{2}	4.0×10^{1}	4.0×10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	3	3	0	3	1	0	0	0	0
	Day 3	2	0	4	2	1	0	0	2	1
	Day 4	=	1	1	=	2	1	2	1	2
	Day 5	=	1	=	=	1	1	2	1	1
	Day 6	=	=	=	=	=	2	1	1	0
	Day 7	=	=	=	=	=	0	=	=	0

(Appendix 1 cont..)

L33

	Concentration	1.33x10^{8}	1.33x10^{7}	1.33x10^{6}	1.33x10^{5}	1.33x10^{4}	1.33x10^{3}	1.33x10^{2}	1.33x10^{1}	1.33x10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	4	2	1	1	1	0	0	0	0
	Day 3	1	3	3	1	2	0	1	0	0
	Day 4	=	=	1	3	1	2	1	0	0
	Day 5	=	=	=	=	0	3	1	3	0
	Day 6	=	=	=	=	1	=	0	2	0
	Day 7	=	=	=	=	=	=	0	=	0

L71

	Concentration	1.33x10^{8}	1.33x10^{7}	1.33x10^{6}	1.33x10^{5}	1.33x10^{4}	1.33x10^{3}	1.33x10^{2}	1.33x10^{1}	1.33x10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	1	0	0	0	0	0	0	0	0
	Day 3	4	3	4	2	2	1	0	0	0
	Day 4	=	2	1	2	1	2	1	1	0
	Day 5	=	=	=	1	1	1	3	2	1
	Day 6	=	=	=	=	0	0	1	0	1
	Day 7	=	=	=	=	1	1	=	2	0

L25

	Concentration	1.6x10^{8}	1.6x10^{7}	1.6x10^{6}	1.6x10^{5}	1.6x10^{4}	1.6x10^{3}	1.6x10^{2}	1.6x10^{1}	1.6x10^{0}
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	2	0	0	0	0	0	0	0	0
	Day 3	3	5	3	2	0	1	0	0	0
	Day 4	=	=	2	2	3	3	2	0	0
	Day 5	=	=	=	1	2	1	3	3	1
	Day 6	=	=	=	=	=	=	=	1	1
	Day 7	=	=	=	=	=	=	=	0	0

Appendix 1 cont..)

L72

	Concentration	$1.33\text{x}10^{8}$	$1.33\text{x}10^{7}$	$1.33\text{x}10^{6}$	$1.33\text{x}10^{5}$	$1.33\text{x}10^{4}$	$1.33\text{x}10^{3}$	$1.33\text{x}10^{2}$	$1.33\text{x}10^{1}$	$1.33\text{x}10^{0}$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	1	1	0	0	0	0	0	0	0
	Day 3	4	4	3	1	1	0	0	1	0
	Day 4	=	=	2	1	1	0	1	0	0
	Day 5	=	=	=	1	1	3	1	3	0
	Day 6	=	=	=	0	1	1	2	1	0
	Day 7	=	=	=	1	1	0	0	=	0

L42

	Concentration	$1.47\text{x}10^{8}$	$1.47\text{x}10^{7}$	$1.47\text{x}10^{6}$	$1.47\text{x}10^{5}$	$1.47\text{x}10^{4}$	$1.47\text{x}10^{3}$	$1.47\text{x}10^{2}$	$1.47\text{x}10^{1}$	1.47x10
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	2	0	0	0	0	0	0	0	0
	Day 3	3	5	1	2	1	1	0	0	0
	Day 4	=	=	4	2	0	3	0	0	0
	Day 5	=	=	=	1	2	1	3	0	0
	Day 6	=	=	=	=	2	=	1	0	0
	Day 7	=	=	=	=	=	=	0	0	1

L65

	Concentration	$2.7\text{x}10^{8}$	$2.7\text{x}10^{7}$	$2.7\text{x}10^{6}$	$2.7\text{x}10^{5}$	$2.7\text{x}10^{4}$	$2.7\text{x}10^{3}$	$2.7\text{x}10^{2}$	$2.7\text{x}10^{1}$	$2.7\text{x}10^{0}$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	0	0	0	0	0	0	0	0	0
	Day 3	5	3	1	1	0	1	0	1	0
	Day 4	=	1	2	3	3	3	2	0	1
	Day 5	=	1	2	0	1	1	2	2	0
	Day 6	=	=	=	0	0	=	1	0	0
	Day 7	=	=	=	1	0	=	=	0	1

(Appendix 1 cont..)

L37

	Concentration	$1.3x10^9$	$1.3x10^8$	$1.3x10^7$	$1.3x10^6$	$1.3x10^5$	$1.3x10^4$	$1.3x10^3$	$1.3x10^2$	$1.3x10^1$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	1	1	1	0	1	0	0	0	0
	Day 3	2	3	3	0	1	0	1	0	0
	Day 4	2	1	1	5	3	1	0	0	0
	Day 5	=	=	=	=	=	2	1	2	0
	Day 6	=	=	=	=	=	2	3	1	0
	Day 7	=	=	=	=	=	=	=	0	1

L24

	Concentration	$2.53x10^8$	$2.53x10^7$	$2.53x10^6$	$2.53x10^5$	$2.53x10^4$	$2.53x10^3$	$2.53x10^2$	$2.53x10^1$	$2.53x10^0$
No. dead	Day 1	0	0	0	0	0	0	0	0	0
	Day 2	1	1	0	1	0	0	0	0	0
	Day 3	4	4	2	1	0	0	0	0	0
	Day 4	=	=	2	2	2	2	0	0	0
	Day 5	=	=	1	1	2	2	1	2	0
	Day 6	=	=	=	=	0	0	2	1	0
	Day 7	=	=	=	=	1	1	0	1	0

<u>NB</u>: = means that all the number injected have died

Concentration is given as organisms/ml.

APPENDIX 2: MIT AND SEROLOGICAL RESPONSE FOR COCKERELS VACCINATED WITH S. GALLINARUM STRAINS CN 180 AND L46

	1 Week			2 Weeks			4 Weeks			5 Weeks			7 Weeks			9 Weeks		
Bird No	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA
#12	16.15	−ve	32	20.16	−ve	-	-	−ve	64	78.7	−ve	128	-	−ve	-	-	−ve	-
#15	3.58	−ve	32	-	−ve	32	NI	−ve	128	-	−ve	-	-	−ve	128	56.13	−ve	64
#17	0.78	−ve	32	-	−ve	64	NI	−ve	64	-	−ve	-	-	−ve	-	NI	−ve	32
# 8	NI	−ve	32	-	−ve	16	-	−ve	256	61.01	−ve	128	-	−ve	64	-	−ve	128
#19	7.61	−ve	64	29.66	−ve	64	3.93	±	256	47.22	−ve	64	-	−ve	64	65.08	−ve	64
#21	19.05	−ve	32	31.61	−ve	128	14.1	−ve	128	67.55	−ve	64	-	−ve	64	64.37	−ve	128
#23	5.74	−ve	128	-	−ve	64	-	±	32	72.87	−ve	256	-	−ve	128	73.66	−ve	128
#10	26.52	−ve	16	16.78	±	32	-	+ve	1024	72.42	+ve	1024	-	+ve	2048	79.16	+ve	1024
#20	37.81	−ve	64	34.18	±	64	-	±	128	67.72	−ve	64	-	−ve	256	-	−ve	256

(Appendix 2 cont..)

	13 Weeks			17 Weeks			21 Weeks			37 Weeks		
Bird No	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA	% migration inhibition	RWBPT	IHA
#12	-	-ve	-	-	-ve	512	-	-ve	16	-	-ve	128
#15	-	-ve	64	NI	-ve	256	-	-ve	128	24.48	-ve	2048
#17	NI	-ve	-	NI	-ve	256	-	-ve	-	77.13	-ve	256
# 8	66.92	-ve	64	8.02	-ve	256	-	-ve	128	NI	-ve	512
#19	46.53	-ve	128	45.5	-ve	128	51.71	-ve	512	-	-ve	256
#21	82.77	-ve	256	-	-ve	512	35.05	-ve	512	14.3	-ve	1024
#23	95.73	-ve	512	19.89	-ve	131072	43.28	-ve	256	29.35	-ve	512
#10	44.69	+ve	512	61.35	+ve	1024	-	+ve	256	80.76	+ve	1024
#20	61.09	-ve	64	NI	-ve	256	44.33	-ve	128	-	-ve	64

<u>KEY</u>

- means "not done" or"set up spoilt"

NI means "no inhibition."

+ve means "positive"

-ve means "negative"

± means "suspiciously positive"

APPENDIX 3: COMPOSITE DATA ON THE SEROLOGY, MIT, LIVER AND SPLEEN HYPERTROPHY INDICES AND LIVER BACTERIAL COUNTS FOR BOTH CHALLENGED CONTROLS AND VACCINATED BIRDS

Day Post-Challenge	Group	Bird No.	RWBPT	% migration inhibition	IHA Titre	Liver hypertrophy Index	Spleen hypertrophy Index	Overall liver counts.
1ST CHALLENGE: 4 WEEKS POST-VACCINATION								
1	Unchallenged controls	A	-ve	NI		=	=	sterile
		B	-ve	NI	64	=	=	sterile
	Challenged controls	C	-ve	=	64	NH	1.4	NG
		D	-ve	NI	64	NH	1.2	$3.2x10^4$org/g
	CN 180 - vaccine	16	+ve	NI	128	NH	1.5	NG
		33	+ve	=	32	NH	1.1	NG
	L46 - vaccine	38	+ve	=	512	NH	1.3	NG
		22	+ve	30.93	512	NH	1.3	NG
2	Challenged controls	E	-ve	NI	128	NH	NH	NG
		F	-ve	NI	=	NH	NH	NG
	CN 180 - vaccine	48	-ve	1.85	256	1.04	1.4	NG
		49	+ve	=	512	NH	NH	NG
	L46 - vaccine	4	±	27.07	64	NH	NH	NG
		43	+ve	6.67	512	NH	1.4	NG
3	Challenged controls	G	-ve	29.67	128	NH	1.2	$8.0x10^2$org/g
		H	-ve	NI	32	NH	1.2	$1.84x10^4$ org/g
	CN 180 - vaccine	30	+ve	45.81	512	NH	NH	$1.0x10^2$org/g
		40	-ve	40.59	256	NH	1.2	$1.2x10^3$org/g
	L46 - vaccine	45	+ve	48.54	256	NH	NH	NG
		36	+ve	11.92	4096	NH	NH	NG
6	Challenged controls	J	-ve	49.05	64	1.09	1.3	NG
		K	-ve	59.37	64	1.13	1.3	$1.0x10^2$org/g
	CN 180 - vaccine	42	-ve	50.01	64	NH	2.0	NG
		35	±	65.47	64	1.22	1.5	$2.0x10^3$org/g
	L46 - vaccine	47	+ve	54.68	16384	1.09	NH	NG
		11	-ve	50.01	32	1.04	1.1	$1.76x10^4$org/g

APPENDIX 3 (Cont..)

Day Post-Challenge	Group	Bird No.	RWBPT	% migration inhibition	IHA Titre	Liver hypertrophy index	Spleen hypertrophy Index	Overall Liver counts
2ND CHALLENGE: 8 WEEKS POST-VACCINATION								
1	CN 180 - vaccine	94	-ve	86.48	64	NH	NH	4.4x10^{3}org/g
		95	-ve	94.12	128	NH	NH	NG
	L46 - vaccine	3	-ve	87.43	128	NH	NH	NG
		5	-ve	83.84	64	NH	1.02	2.0x10^{3}org/g
2	CN 180 - vaccine	93	+ve	60.36	64	1.2	NH	NG
		100	+ve	89.23	512	NH	NH	NG
	L46 - vaccine	37	+ve	77.23	64	NH	1.19	200 org/g
		29	+ve	92.93	256	NH	1.19	5.12x10^{4}org/g
3	CN 180 - vaccine	99	+ve	86.24	128	NH	1.08	NG
		96	+ve	88.13	128	NH	NH	200 org/g
	L46 - vaccine	44	-ve	78.79	64	NH	1.08	NG
		13	+ve	70.23	4096	1.04	NH	NG
6	CN 180 - vaccine	97	+ve	79.08	256	NH	1.02	500 org/g
		86	+ve	85.14	256	NH	1.02	200 org/g
	L46 - vaccine	34	-ve	88.5	128	1.04	NH	NG
		31	+ve	77.52	128	NH	NH	200 org/g
3RD CHALLENGE: 13 WEEKS POST-VACCINATION								
1	CN 180 - vaccine	83	+ve	=	128	NH	NH	NG
		82	-ve	=	64	NH	NH	NG
	L46 - vaccine	14	-ve	=	128	NH	NH	NG
		80	+ve	=	256	NH	NH	200 org/g
2	CN 180 - vaccine	79	+ve	=	256	NH	NH	200 org/g
		81	-ve	=	256	NH	NH	200 org/g
	L46 - vaccine	2	-ve	=	64	1.04	NH	NG
		77	-ve	=	32	NH	NH	NG

APPENDIX 3 (Cont...)

Day Post-Challenge	Group	Bird No.	RWBPT	% migration inhibition	IHA Titre	Liver hypertrophy Index	Spleen hypertrophy Index	Overall Liver counts.
5	CN 180 - vaccine	73	+ve	=	512	1.39	NH	$4x10^2$ org/g
		74	+ve	=	256	1.39	1.65	$4x10^2$ org/g
	L46 - vaccine	75	+ve	=	256	1.3	NH	$4x10^2$ org/g
		76	+ve	=	256	1.7	1.14	$8x10^2$ org/g
6	CN 180 - vaccine	69	+ve	39.2	512	1.04	1.08	NG
		70	-ve	52.55	128	NH	NH	$1.2x10^3$org/g
	L46 - vaccine	72	+ve	36.98	512	1.26	NH	200 org/g
		6	+ve	60.84	512	NH	NH	$2x10^2$ org/g
4TH CHALLENGE: 17 WEEKS POST-VACCINATION								
4	CN 180 - vaccine	66	-ve	=	1024	NH	NH	200 org/g
		67	-ve	29.13	2048	1.13	NH	200 org/g
	L46 - vaccine	63	-ve	NI	256	1.26	NH	$1.2x10^3$org/g
		64	+ve	52.03	128	1.09	NH	NG
6	CN 180 - vaccine	36	+ve	16.68	4096	1.52	1.08	$8.192x10^5$org/g
		47	+ve	49.65	64	1.17	1.14	$2x10^2$ org/g
	L46 - vaccine	37	+ve	59.8	256	1.17	NH	NG
		40	+ve	43.06	256	1.09	NH	NG
10	CN 180 - vaccine	99	+ve	50.91	65536	1.13	1.02	NG
		95	+ve	34.03	8192	1.09	NH	200 org/g
	L46 - vaccine	96	+ve	70.83	512	1.26	NH	NG
		97	+ve	26.87	2048	NH	1.02	NG
12	CN 180 - vaccine	62	+ve	57.28	8192	1.17	NH	NG
		60	+ve	24.47	512	1.09	NH	NG
	L46 - vaccine	61	+ve	68.85	8192	1.09	NH	NG
		=						

KEY

+ means "positive"
-ve means "negative"
± means "doubtful"
= means "not done"
NI means "no inhibition"
NH means "no hypertrophy"
NG means "no bacterial growth"

APPENDIX 4: STATISTICAL COMPARISON OF LIVER HYPERTROPHY AND RECOVERY OF BACTERIA FROM THE RESPECTIVE LIVERS

CONTROLS

	NG	G	Total
H	1	1	2
NH	3	3	6
TOTAL	4	4	8

$\chi^2=1.0$

CN 180 - VACCINATED BIRDS

	NG	G	Total
H	6	7	13
NH	9	10	19
TOTAL	15	17	32

$\chi^2=0.25$

L46 - VACCINATED BIRDS

	NG	G	Total
H	9	5	14
NH	11	6	17
TOTAL	20	11	31

$\chi^2=2.25$

KEY

H means "hypertrophy"
NH means "no hypertrophy"
NG means "no bacterial growth"
G means "bacterial growth"

APPENDIX 5: STATISTICAL COMPARISON OF SPLEEN HYPERTROPHY AND RECOVERY OF BACTERIA FROM THE RESPECTIVE LIVERS

CONTROLS

	NG	G	Total
H	2	4	6
NH	2	0	2
TOTAL	4	4	8

$\chi^2 = 0.67$

CN 180 - VACCINATED BIRDS

	NG	G	Total
H	7	7	14
NH	8	10	18
TOTAL	15	17	32

$\chi^2 = 0.07$

L46 - VACCINATED BIRDS

	NG	G	Total
N	5	5	10
NH	15	6	21
Total	20	11	31

$\chi^2 = 5.0$

KEY

H means "hypertrophy"
NH means "no hypertrophy"
NG means "no bacterial growth"
G means "bacterial growth"

APPENDIX 6: LIVER HYPERTROPHY VS SPLEEN HYPERTROPHY IN DETECTING POSITIVE CASES i.e. CASES WHERE THERE WAS NO GROWTH IN THE RESPECTIVE LIVERS

CONTROLS

	SH	SNH	Total
LH	0	1	1
LNH	1	2	3
Total	1	3	4

$\chi^2=0.0$

CN 180 - VACCINATED BIRDS

	SH	SNH	Total
LH	3	3	6
LNH	4	5	9
Total	7	8	15

$\chi^2=0.14$

L46 - VACCINATED BIRDS

	SH	SNH	Total
LH	0	9	9
LNH	5	6	11
Total	5	15	20

$\chi^2=1.14$

OVERALL BREAKDOWN

	SH	SNH	Total
LH	3	13	16
LNH	10	13	23
Total	13	26	39

$\chi^2=0.39$

KEY

LH means "liver-hypertrophy"
LNH means "liver-no hypertrophy"
SH means "spleen-hypertrophy"
SNH means "spleen-no hypertrophy"

APPENDIX:7. ANTIBIOTIC SENSITIVITIES OF THE 35 S. GALLINARUM ISOLATES SURVEYED

Isolate	Co-Trimoxazol (SXT)	Nitrofurantoin (F)	Nalidixic acid (NA)	Gentamycin (CN)	Chloramphenicol (C)	Compound Sulphonamide (S3)	Teracycline (TE)		Erythromycin (E)	Ampicillin (AMP/PN)		Cloxacillin (OB)	Penicillin G (P)	Streptomycin (S)		Furazolidone (FR)	Neomycin (N)
	(25 μg)	(200 μg)	(30 μg)	(30 μg)	(30 μg)	(200 μg)	(10μg)	(100μg)	(10 μg)	(2μg)	(25μg)	(5 μg)	(1.5 i.u)	(10μg)	(25μg)	(15 μg)	(10 μg)
L21	30	36	22	34	26	10	28	40	15	14	38	9	9	9	12	30	26
L32	32	34	20	32	26	9	26	40	16	14	34	9	9	9	10	34	28
L30	32	34	16	42	28	26	26	34	18	12	30	9	9	10	14	-	-
L52	39	32	22	40	28	24	26	32	22	18	40	9	9	12	16	-	-
L12	25	32	14	32	22	9	26	38	16	18	36	9	9	9	10	-	-
L39	34	34	18	42	28	18	28	36	18	14	32	9	9	9	12	-	-
L7	31	32	14	38	14	20	22	36	16	10	34	9	9	9	12	-	-
L24	32	38	18	32	28	9	26	42	18	20	40	9	9	9	12	-	-
L17	30	36	18	24	26	9	28	42	18	18	40	9	9	9	16	-	-
L64	26	33	20	34	26	10	28	44	18	16	38	9	9	9	16	-	-
L38	31	44	36	40	30	12	30	40	20	24	40	9	9	9	22	-	-
L40	33	32	18	34	26	9	26	38	16	18	38	9	9	9	10	-	-
L27	31	34	16	34	24	9	28	40	18	18	40	9	9	9	12	-	-
L46	29	34	18	32	20	24	22	36	18	9	28	9	9	9	9	-	-
L34	28	34	20	31	28	9	28	40	18	24	40	9	9	9	9	-	-
L31	38	34	24	40	26	30	26	38	14	16	38	9	9	10	20	34	28
L43	34	34	16	36	26	9	26	40	18	16	36	9	9	9	12	-	-
L59	29	38	20	32	26	12	28	38	18	18	38	9	9	9	16	-	-
L18	30	32	24	36	28	9	28	36	16	18	36	9	9	9	18	-	-
L61	38	36	26	40	24	30	22	34	14	14	34	9	9	20	28	-	-
L49	32	28	14	38	24	24	22	34	16	16	30	9	9	9	10	-	-
L56	37	36	9	40	22	22	10	20	16	12	34	9	9	9	16	34	27
L60	28	38	20	36	26	9	28	42	16	20	34	9	9	9	16	-	-
L66	37	38	20	40	24	30	26	38	18	12	36	9	9	PS12	16	-	-
L44	29	36	18	34	30	9	28	40	18	18	40	9	9	9	9	-	-
L41	33	30	12	36	22	20	26	30	20	9	32	9	9	9	12	-	-
L29	36	34	18	34	24	9	28	40	18	18	40	9	9	9	10	-	-
L73	PS14	28	PS14	22	17	9	PS16	26	PS12	PS14	28	9	9	PS14	PS18	-	-
CN180	20	32	13	22	27	PS17	29	40	15	12	32	9	9	9	17	-	-
L68	-	-	-	-	18	9	18	-	-	9	-	-	9	17	-	32	30
L69	-	-	-	-	26	9	20	-	-	15	-	-	9	9	-	32	26
L70	-	-	-	-	24	9	20	-	-	14	-	-	9	11	-	30	26
L15	28	42	22	34	26	9	32	42	18	16	40	9	9	9	12	35	30
L6	-	-	-	-	26	PS16	24	-	-	14	-	-	9	PS12	-	26	26
E.Coli	29	27	27	25	23	15	-	37	9	-	27	9	9	-	23	-	-
Staph.	39	33	19	31	31	33	-	39	39	-	40	39	41	-	27	-	-

KEY: (i) Measurements are given in millimetres (inhibition zone diameter)
(ii) Disc diameter = 9mm.
(iii) PS means "partial sensitivity"
(iv) - means "not done"

APPENDIX 8:

Key: (i) Measurements are given in millimetres (inhibition zone diameter)

(ii) Disc diameter = 6mm

(iii) Ps means "partial sensitivity"

APPENDIX: 8 DISINFECTANT SENSITIVITIES OF THE 30 S. GALLINARUM ISOLATES SURVEYED

	LYSOL (Ly)				PYNOL 5 (Fy)				KEROL (KR)					BIODAN (FD)					BROMOSEPT (DN)					MUNICIPAL FLUID (MN)				
	0.5%	1%	2%	4%	1%	2%	5%	10%	0.17%	0.33%	1.0%	1.7%	3.3%	0.6%	1.8%	2.5%	4%	6%	0.01%	0.1%	0.5%	1%	2%	0.3%	0.7%	2%	10%	100%
L21	6	6	10	14	6	8	9.5	11	6	7	11	11.5	15	6	6	7	9	13	12	22	26	38	28	6	6	6	6	11
L32	6	9	10	14	6	8	9	10	6	9	11	12	13	6	6	7	10	14	14	21	26	26	22	6	6	6	6	12
L30	6	8	10	14	6	6	6	7	6	8	10	11	13.5	6	6	8	11	16.5	12	15	19	18	18.5	6	6	6	6	12
L52	6	6	9	12	6	6	8.5	12	6	10	12	13	15	6	6	7	10.5	13.5	12	15	17	17.5	17.5	6	6	6	6	11
L12	6	6	9	11	6	8	9.5	10.5	6	6	8	10	13	6	6	7	8	10	11	13	16	18	18	6	6	6	6	11
L39	6	7	9.5	11	6	7.5	8	9	6	9	12	13	15	6	6	8	10.5	13	12	13	19	23	23	6	6	6	11	13
L7	6	6	10	14	6	6	8	9.5	6	7.5	9.5	10.5	12.5	6	6	9	11.5	17	12	19	22	20	36	6	6	6	6	11
L24	6	6	9.5	13	6	6	6	7	6	8	11	11.5	13.5	6	6	7	9	11.5	12	13	26	38	22	6	6	6	6	11
L17	6	8	11	14	6	6	9	10	6	8	11	11.5	13	6	7	9.5	14	18	12	14.5	17	13.5	19	6	6	6	6	12
L64	6	6	9.5	14	6	6	8	10	6	9	10.5	12	15	6	6	6.5	10	13	14	22	23	22	28	6	6	6	16	12
L38	6	6	9.5	12	6	6	9	11	6	8	10.5	12	15	6	6	9	12	14	7	16	20	24	21	6	6	6	6	11
L40	6	9	9	12	6	9	10.5	12.5	6	6	9	11	12.5	6	6	8	10	14	12	16	19	20	24	6	6	6	6	12
L27	6	6	9	12	6	6	7.5	10	6	6	8	10.5	13	6	6	8	12	16	13	15	24	27	23	6	6	6	6	11
L46	6	6	11	13	6	6	8	9	6	8	10	10	13	6	6	7	11.5	16	12	14	18	19	19	6	6	6	6	11
L34	6	7	10	13.5	6	6	7.5	8	6	6	8	9	12	6	7.5	8.5	10.5	16	14	16	18	21	18.5	6	6	6	6	11
L31	6	8	9.5	11.5	6	9	13	14	6	9	11	12	14	6	6	9	11	14	12	23	24	20	18	6	6	6	6	12
L43	6	7	8.5	12	6	6	6	7	6	7	11	12.5	15	6	6	6	8	9.5	11	13	21	23	25	6	6	6	6	12
L59	6	6	9	12	6	6	7	9	6	8	10.5	12	14	6	6	8	11.5	16	13	16.5	16	19	19	6	6	6	6	11
L18	6	6	9	11.5	6	6	8	9	6	7	9	12	13	6	6	7	10	13	10.5	12	22	22.5	19	6	6	6	6	11
L61	6	8	11	13	6	6	7	9	6	7.5	10	11	12	6	6	9.5	11	12	6	11.5	13	13	13	6	6	6	6	11
L49	6	9.5	12	14	6	9	12	16	6	8	10.5	12	13	6	7.5	7	9.5	12.5	22	24	40	46	40	6	6	6	6	11
L15	6	7	11	14	6	6	9	10	6	9	11.5	13	16	6	6	7.5	10	14	12	14	16	17	18.5	6	6	6	12	15
L56	6	7	10	13	6	8	9	10	6	8.5	11	11	13.5	6	6	9	11.5	14	11	15	26	24	23	6	6	6	6	11
L60	6	9.5	11	14	6	6	8	9.5	6	10	13	13.5	14	6	7	8	11.5	15	12	20	26	27	24	6	6	6	6	11
L66	6	7	11	14	6	7.5	9	11	6	7.5	10	11	13	6	7	10.5	11	17	21	22	18	26	22	6	6	6	6	11
L44	6	8.5	10.5	14	6	6	6	7	6	8	11	12	14	6	6	9	10	14	9	12	17	16	17	6	6	6	6	12
L41	6	7	11	13.5	6	6	6	8	6	7.5	9	11	14	6	7	9	13	16.5	10	12	16	14	20	6	6	6	6	12
L29	6	7	10.5	13	6	6	6	6	6	7	9.5	11	11.5	6	8	8	12	15.5	12	22	29	16	14	6	6	6	6	11
L73	PS10	10	13	14	9	10	11	12	8	10	12	13	16	6	7	10	12	13	9	9	10	11	13	6	6	6	6	11
CN180	6	12	23	27	6	6	8	9.5	6	9	11	12	18	6	9	11	14	17	10	10	11	13	16	6	6	6	11	13
E.Coli	6	6.5	10.5	13	6	6.5	8	9	6	7.5	9	12	13	6	7	8.5	10.5	12.5	8	12	18	16	15	6	6	6	11	13
Staph.	8	11	14	16	11	15	20.5	22.5	6	12.5	17	24.5	28.5	6.5	9.5	11	13	14	14	18	25	19	20	6	6	6	11	13