EGG TRANSMITTED IMMUNITY TO *SALMONELLA TYPHIMURIUM* PHAGE TYPE 49 IN THE CHICKEN

Thesis submitted for the degree of Doctor of Philosophy
in the Faculty of Veterinary Medicine, University of Glasgow

by

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October 1989

In the name of Allah
The Compassionate, The Merciful

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ACKNOWLEDGEMENTS

I would very much like to thank the following people for their help in the production of this thesis.

My supervisor Dr. David Taylor for his help, friendly guidance and constant encouragement.

Professor W.F.H. Jarrett, head of the Department of Veterinar y Pathology, for allowing me to undertake these studies in his department.

Mr. A. Bradley and staff for care of some of the chickens reported in this study.

Dr. A. Rycroft for his technical assistance in SDS PAGE.

Mrs Carol MacLay, Miss C. Oldacre and other technical staff of the Department of Veterinary Pathology for their technical assistance.

The staff of the E.M. Section of Department of Veterinary Pathology for their help in the E.M. studies.

Mr A. May and Mr C. Cullen for the preparation of Photographs.

My wife for her patience, encouragement and typing the manuscript.

Dr. I. McCandlish for her help and providing computer facilities to print part of this thesis.

The Government of Islamic Republic of Iran and the University of Shiraz, Shiraz, Iran, for awarding me the scholarship and supporting this work.

Habibollah Dadrast

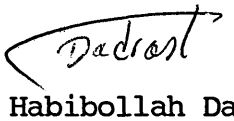
October, 1989

PREFACE

The work described in this thesis was carried out in the Department of Veterinary Pathology, University of Glasgow from October 1986 to October 1989 under the able supervision of Dr. David John Taylor, MA. Vet.MB. PhD. MRCVS.

These studies represent original work carried out by the author, and have not been submitted in any form to any other university. Where use has been made of material provided by others, due acknowledgement has been made in the text.

October, 1989



Habibollah Dadrast

SUMMARY

Salmonella typhimurium phage type 49 which had been isolated from a broiler flock was used at low passage to infect day old specific pathogen free (SPF) chicks. 100% morbidity was observed in chicks directly inoculated with a dose of 0.2 x 10^3 CFU to 2 x 10^3 CFU of this organism or those that were in contact with infected birds. Various degrees of mortality were also observed in different groups of infected chicks. The colonised chicks were carriers of the organism until at least 6 months.

A double antibody indirect hetrogeneous enzyme linked immunosorbent assay (ELISA) was adapted for chickens to study the levels of different immunoglobulins to S. typhimurium in sera, bile, oviductal secretions, egg white and egg yolk from chickens infected with S. typhimurium or vaccinated with killed vaccines.

Surface protein and lipopolysaccharide antigens of S. typhimurium pt. 49 were used to coat the surface of the wells of polystyrene plates. Goat anti-chicken Fc specific antibodies, rabbit anti-goat IgG conjugated with horseradish peroxidase, hydrogen peroxide and ortho-phenylene diamine were the reagents used in this ELISA.

Specific IgA, IgM and IgG directed against S. typhimurium was measured in the sera and eggs of naturally infected chickens. The levels of these antibodies varied among the individuals. IgG ranged from 1:75 to 1:4800, IgM from 1:400 to 1:6400 and IgA from 1:200 to 1:1600 in the sera of 11 chickens examined. Specific IgA (1:1280-1:10240) and IgM (1:40-1:640) were present in the bile of these chickens. Specific IgA and IgM were found in egg white and specific IgG in the egg yolk of infected hens. A correlation was observed between the levels of yolk IgG and that in the serum.

Chicks derived from the eggs of naturally infected hens were protected from systemic infection and gut colonisation was delayed for at least 7 days. This observation suggested that humoral immunity could be largely responsible for protection against salmonellosis.

12 SPF chickens (in 2 groups of 5 hens and 1 cockerel) were vaccinated. One group was vaccinated parenterally and both received oral vaccine. The vaccine for oral vaccination by feed inclusion was formalin killed, sonicated and freeze dried. Parenteral vaccine consisted of a fresh formalin killed suspension of the organism enriched with surface protein antigens and emulsified with Arlacel A and liquid paraffin. Hens were injected subcutaneously 3 times at the age of 19, 23 and 36 weeks.

Specific IgA, IgM and IgG antibodies to S. typhimurium were measured in the sera and eggs of parenterally-orally vaccinated hens. IgG levels of up to 1:76800 were detected in the sera and levels of up to 1:40960 were found in the egg yolk. Anamnestic effects were observed after the 2nd and 3rd injections. The orally vaccinated hens failed to produce specific IgG in their sera or eggs. Specific IgA (up to 1:1280) and IgM (up to 1:640) were found in the bile and egg white of both groups.

Chicks derived from the eggs of parenterally-orally vaccinated hens showed specific IgG (1:38400), IgA (1:3200) and IgM (1:1600) in their sera. These levels declined with age. The transfer of maternal IgA and IgM antibodies to the sera of the chick has not been demonstrated previously.

Immunoglobulins were isolated from the egg white and egg yolk of the parenterally-orally vaccinated hens and injected into the yolk and allantoic cavity of embryonated eggs and also into the egg albumin prior to incubation. Injection into the

egg yolk provided the best results in terms of hatching (80%) and conferred most protection against the organism to chicks after hatching. Mortality was prevented and colonisation of the gut was delayed in the chicks derived from eggs into the yolk of which IgG had been artificially transferred on day 10 of incubation.

CHAPTER 1

REVIEW OF THE LITERATURE ON AVIAN SALMONELLOSIS, AVIAN DEFENCE SYSTEMS, SALMONELLA VACCINE AND EGG TRANSMITTED IMMUNITY.

1 - INTRODUCTION

Salmonellosis is a disease of world wide economic importance in humans and animals (Clarke and Gyles, 1986). It is a disease of all animals especially food animals and these constitute a vast reservoir of the disease for humans. Salmonellosis of animal origin is an important cause of human food poisoning and the cost that it imposes upon society is enormous.

Domestic poultry are the largest single reservoir of Salmonella organisms in the human food supply and Salmonella contamination in poultry and poultry products is more frequently reported than in all other animal species (Botes 1965, Williams 1978).

Salmonella infection causes the loss of thousands of lives and the hospitalisation of millions around the world every year. The figure for economic damage and the cost of preventive measures is immeasurable on a world wide basis. As an example, the social cost of salmonellosis in West Germany in 1977 was estimated to be 2512 million German marks, 52% of this was said to be of domestic animal origin and 10% was traced to poultry (Krug 1985). Yule et al. (1988) reported that food borne salmonellosis of poultry origin was the most common form of the infection in Scotland. During the period 1980-1985, 224 outbreaks of poultry-borne salmonellosis affected 2245 persons and killed 12 . They estimated that the annual cost of salmonellosis in man in Scotland was £252776 - £751696 in Scotland. From November 1979 to October 1982 there were 308

outbreaks of Salmonella food poisoning in England and Wales of which 56% was associated with poultry (Watson and Kirby 1985).

Apart from the human side, salmonellosis is a problem of major economic concern in all branches of the food animal industry including poultry. In poultry it is one of the important egg borne diseases and outbreaks of Salmonella infection in hatcheries cause massive economic damage to farmers. In breeding flocks the cost of prevention and eradication is extremely high. Outbreaks of Salmonellosis in valuable breeding stock may terminate breeding operations because of its chronic nature and the difficulty of eradication. In both the poultry meat and egg production industries, remarkable reductions in production and great loss of income occur due to down grading at slaughter and the condemnation of contaminated carcasses and infected eggs.

The annual cost of salmonellosis due to paratyphoid infections alone in poultry is $77 millions in the USA. (Williams 1984). In December 1988 the British government allocated £19 million for compensation payments to egg producers for the slaughter of infected egg laying flocks due to public loss of confidence arising from infection with Salmonella enteritidis phage type 4. The real cost of the damage to farmers and the egg industry has yet to be published.

The Genus Salmonella is a member of the family Enterobacteriaceae and is composed of more than 2000 serologic types each with a specific serotype name (Clarke and Gyles 1986, Tauxe 1988). Some of these are host specific and infect certain species e.g. S. pullorum and S. gallinarum in poultry, S. dublin in cattle, S. abortus-equi in the horse, S. abortus-ovis in sheep, S. cholerae-suis and S. typhisuis in pigs, S. typhi, S. paratyphi, S. schottmuelleri, S. hirschfeldii and S. sendai in man. Others are not adapted to specific animals and cause disease in man and a variety of animals. This group includes the vast majority of Salmonellae and S. typhimurium is

the most frequent cause of the disease. (Clarke and Gyles 1986). Those from the latter group that cause infection in poultry are called the paratyphoid group (Williams 1984) and hereafter this name will be used in the text where applicable.

Various attempts have been made to control and eradicate salmonellosis in all domestic animal species, but little has been achieved. Eradication of specific infections such as S. pullorum and S. gallinarum has been possible in poultry but no similar eradications have been recorded in other domestic animal species (Snoeyenbos 1984). Control measures will be reviewed later in this text under avian salmonellosis below.

The literature on salmonellosis is voluminous and attention will be directed to avian salmonellosis with special reference to S. typhimurium. This review will also cover the various aspects of the immune system of the fowl and the different immunoglobulins (Ig) and other defence mechanisms which are under close consideration by those working on the prevention of Salmonella infections.

2 - AVIAN SALMONELLOSIS

In this section the epidemiology of poultry salmonellosis, growth requirements of Salmonella and its morphology on different biological media, pathogenicity and the factors affecting the virulence of Salmonella in the chicken are discussed.

A) History, incidence and distribution

The earliest work on Salmonella gallinarum was carried out by Kelin in 1889 (Pomeroy 1984) and the aetiological agent of pullorum disease was discovered by Rettger in 1899 (Snoeyenbos 1984). The first non host specific Salmonella (Paratyphoid) was isolated by Moore in 1895 from domestic poultry (Williams 1984). Since then there were reports of paratyphoid infection

from all corners of the world and Salmonellae from avian sources have played an important role in human and other animal infections (Williams 1984, Jephcott 1984). Individual serotypes may be prevalent in some areas, but in general 70% of the disease outbreaks are due to 10 to 12 serotypes (Williams 1984). Clarke and Gyles (1986) stated that out of 2000 serotypes (species) of Salmonella fewer than 50 occur frequently in disease outbreaks. S. typhimurium is the most important of these serotypes. In the USA it was the organism most frequently isolated from human outbreaks from 1976 to 1986 (Tauxe 1988).

The annual salmonellosis report in the U.K. for 1986 (Anon. 1986) shows that S. typhimurium was the most prevalent serotype in all classes of poultry except turkeys during the period of 1976 to 1986, and accounted for 14% of fowl isolates, 4% of turkey isolates, 53% of duck and goose isolates and 42% of game bird isolates. The report also clearly showed that this serotype was most important among the Salmonella isolates from other animals. Fig. 1 shows the Salmonella isolations during this period from all animals (including birds) and birds and compares total S. typhimurium incidents to total incidents of all serotypes.

Using data from the same source, the incidence of Salmonella isolation from different classes of bird during 76-86 is plotted in Fig. 2. The report also concludes that of all phage types of S. typhimurium, phage type 49 was the most prevalent during the whole period with an increasing trend and peak in 1986. The next most common phage types after 49 were phage types 99, 8 and 141 respectively. Fig. 3 shows the incidence of each phage type during the whole period in comparison to the incidence of all phage types of S. typhimurium. The prominence of this serotype led to its choice as the subject of this study.

FIG. 1:

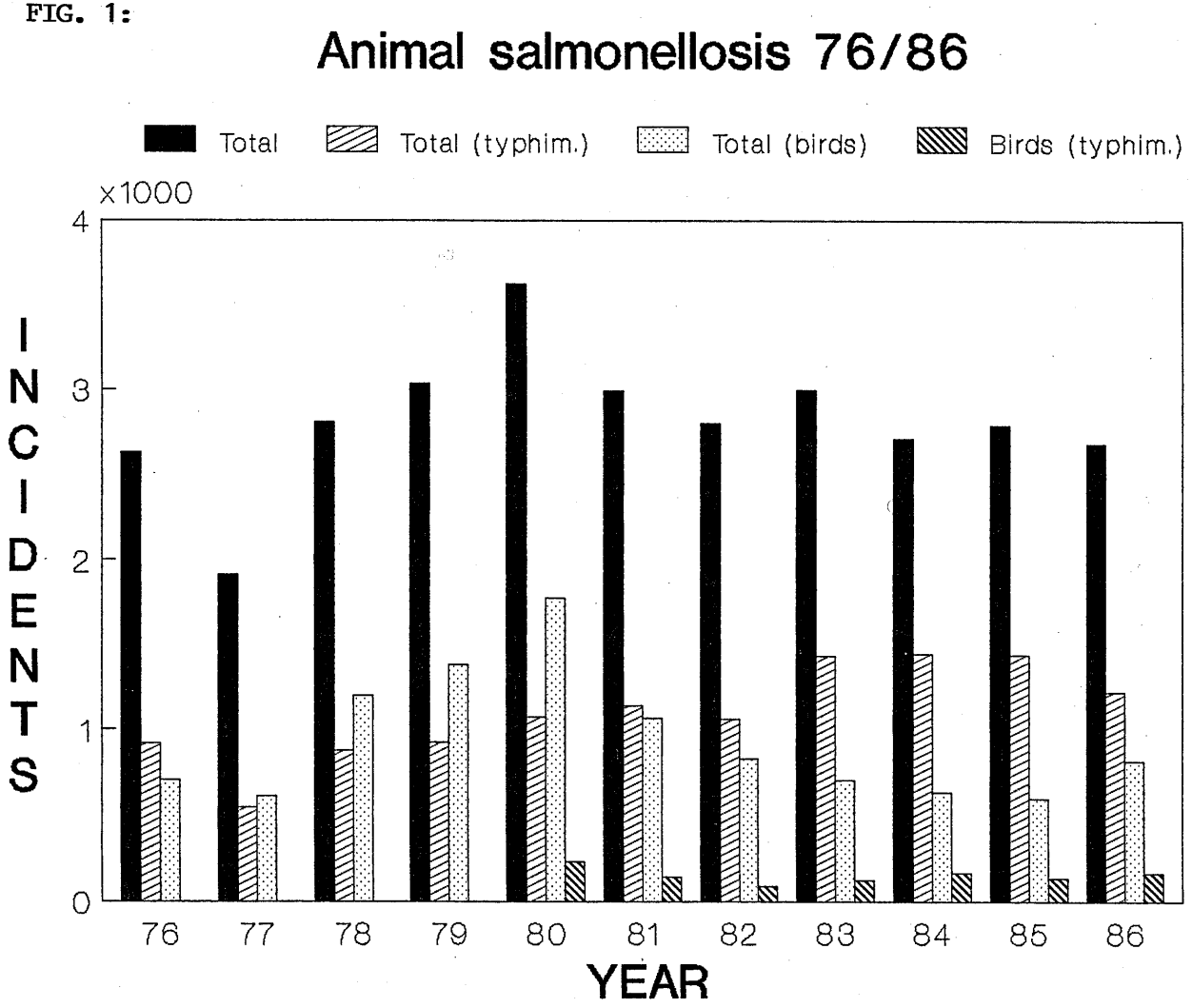

Animal salmonellosis 76/86
Total
Total (typhim.)
Total (birds)
Birds (typhim.)
x1000
4
3
2
1
0
INCIDENTS
76
77
78
79
80
81
82
83
84
85
86
YEAR

FIG. 2:

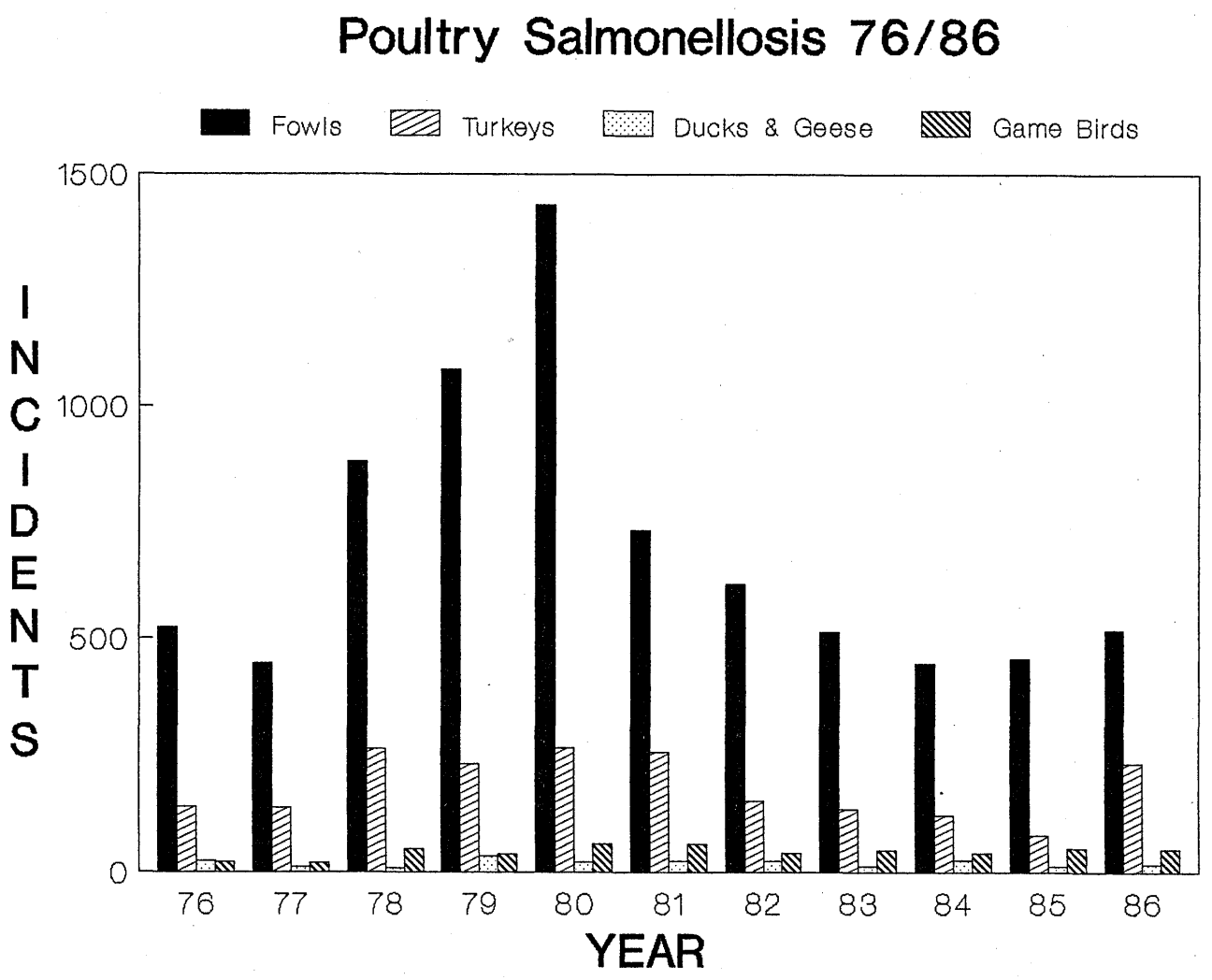
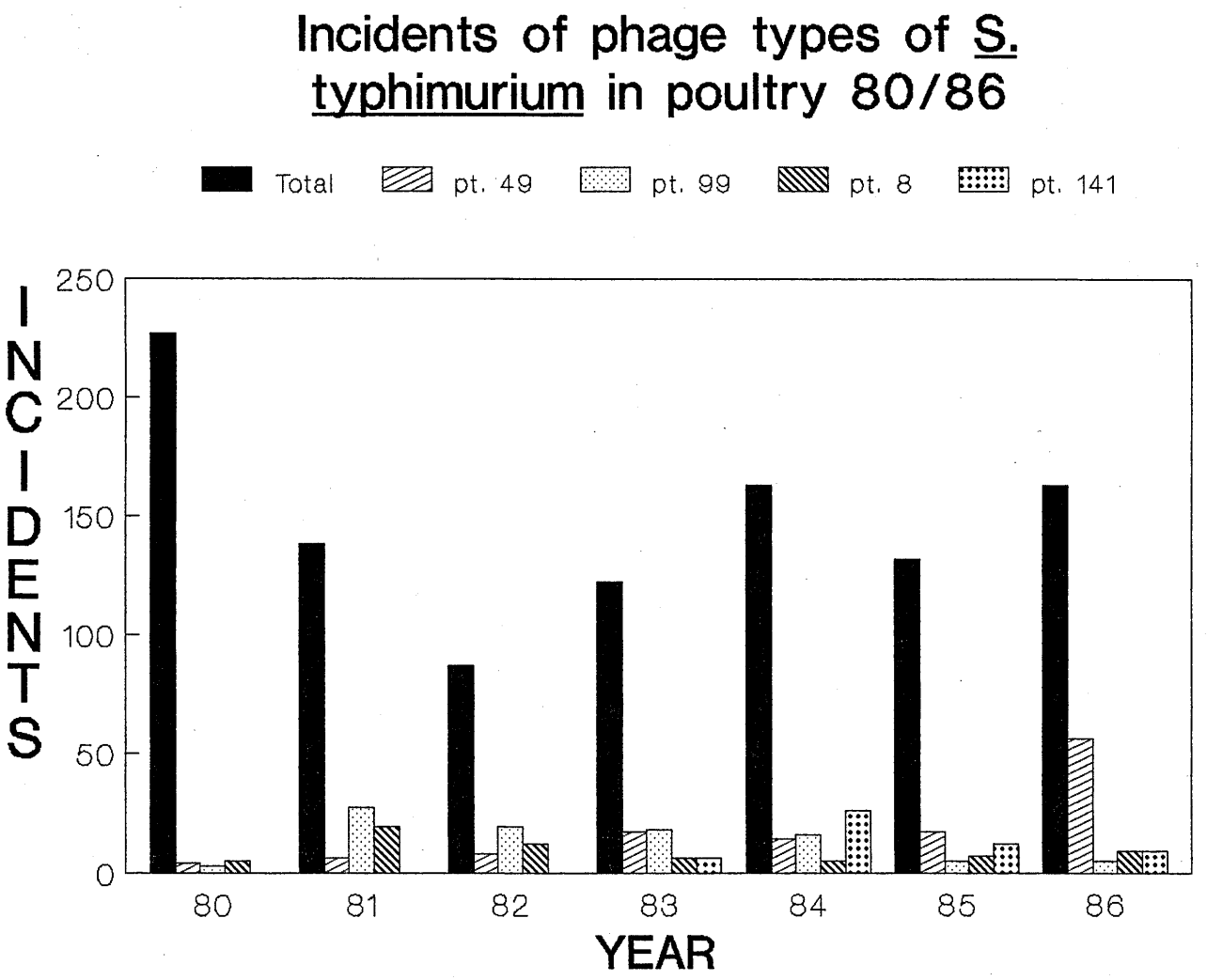

Poultry Salmonellosis 76/86
Fowls
Turkeys
Ducks & Geese
Game Birds
1500
1000
500
0
INCIDENTS
76
77
78
79
80
81
82
83
84
85
86
YEAR

FIG. 3:


Incidents of phage types of S. typhimurium in poultry 80/86
Total
pt. 49
pt. 99
pt. 8
pt. 141
INCIDENTS
250
200
150
100
50
0
80
81
82
83
84
85
86
YEAR

B) Aetiology

S. pullorum and S. gallinarum, the causative agents of pullorum and typhoid diseases in poultry are host specific and non motile with many specific characters in vivo and in vitro which distinguish them from the more widespread paratyphoid group. From this point forward this review will therefore concentrate on the paratyphoid group of Salmonella. The general characters of the paratyphoid group are reviewed below using the criteria of Edwards and Ewing (1962) and Williams (1984).

a) Cellular morphology

All Salmonellae belonging to the paratyphoid group are Gram-negative, non sporing bacilli with dimensions of 0.4-0.6 by 1-3 um. Sometimes they form short filaments. They are motile with peritrichous flagella and fimbriae.

b) Biochemical properties and growth requirements

Salmonella is a facultatively anaerobic organism growing readily on simple media. The optimum growth temperature is 37^{o}C (Williams 1984). Salmonella can grow at 43^{o}C and this incubation temperature can be used as an advantage to inhibit the growth of competing microflora especially Proteus bacteria (Pietzch 1985). Incubation at 44^{o}C inhibits the production of flagella (Parton 1975). The stock culture can remain viable in paraffin-corked nutrient agar for many years (Williams 1984). According to Edwards and Ewing (1962), Williams et al. (1980) and Williams (1984) the members of paratyphoid group have the following biochemical characters:

Dextrose, mannitol, maltose, sorbitol and usually dulcitol are fermented with gas production. They do not ferment lactose, sucrose, salicin or adonitol and may or may not ferment inositol. They do not produce urease and indole. Gelatine is rarely liquefied and Simmon's citrate is usually

utilised. They produce hydrogen sulphide and reduce nitrate. They decarboxylate lysine, arginine and ornithine. Phenyl alanine deaminase activity and malonate utilisation is negative. They are motile and positive in the motility test. The methyl red test is positive. The potassium cyanide test and acetoin production (Voges Proskauer test)are both negative.

c) Isolation media and colonial morphology

Selective and differential media have been devised for the isolation of Salmonellae from different types of samples using the biochemical characters described above. Some of the media that are used routinely and recommended by most research workers are given below:

Tetrathionate broth: This is a selective enrichment medium for the isolation of Salmonellae from faeces, sewage etc. Salmonellae can reduce tetrathionate and grow in this medium (Oxoid 1982). Fresh samples of faeces, organs and meat or its products from animals infected with Salmonella may be inoculated into tetrathionate broth and competing bacteria may be eliminated (Pietzch 1985). Two disadvantages of this medium are that:

a) It is unsuitable for isolation of S. abortus-ovis, S. cholera-suis and S. paratyphi (MAFF 1984).

b) Members of the Proteus group can grow in it by the reduction of tetrathionate and consequently can impair the value of this medium for Salmonella isolation. This problem can be over come by the addition of Novobiocin to the medium (Oxoid Manual 1982).

Brilliant Green (BG) Agar: BG agar is a highly selective medium for Salmonella isolation and inhibits the growth of lactose and sucrose fermenters such as coliforms etc. It is suitable for isolation of Salmonella from food, faeces and dairy products

and it is recommended for isolation of Salmonella from avian samples (Williams et al. 1980).

Salmonella colonies after 24 hours incubation on BG agar are usually transparent pink to deep fuchsia, surrounded by reddened medium. In the presence of lactose fermenting colonies on the medium, Salmonella colonies are brownish with little change in the colour of the medium (Williams et al. 1980). Lactose fermenters are inhibited and, if they grow at all, produce yellow green colonies surrounded by a yellow green zone (Oxoid Manual 1982).

Salmonella Shigella (SS) Agar: This is a differential, selective medium for isolation of Salmonella and Shigella species. A modified form with lower bile salt concentrations and slightly higher pH (7.3) is preferred and permits a better growth of these organisms. Salmonella colonies on this medium are 1-2 mm and transparent usually with black centres after 24 hours incubation at 37^{o}C (Oxoid Manual 1982).

Desoxycholate Citrate Agar (DCA) : This is a selective medium used for the isolation of intestinal pathogens. The growth of Proteus and coliform species is usually inhibited on this medium. After 24 hours incubation at 37^{o}C, lactose fermenting organisms produce pink colonies usually surrounded by a precipitated zone of desoxycholic acid. Salmonellae appear as 1 mm, slightly opaque and dome shaped colonies. If incubated up to 48 hours the colonies grow in diameter (2-4 mm) and show a central black spot (MAFF 1984, Oxoid Manual 1982).

MacConkey Agar: This is a selective medium used for the isolation and differentiation of intestinal bacteria and pathogens from coliforms (Oxoid Manual 1982). The presence of lactose in this medium differentiates lactose fermenters from non lactose fermenters. Salmonellae from the paratyphoid group grow very well on this medium, producing smooth colourless (non lactose fermenting) round, shiny, 2 mm colonies after 18-24 hours incubation. Lactose fermenters appear red on this medium

(MAFF 1984).

Sheep blood agar: This is a non selective medium used for the culture of pathogenic and non pathogenic bacteria. Blood is added to the agar base to determine the haemolytic reaction of some of the bacteria. Salmonella growth on this medium appears as non haemolytic, smooth, grey, 2-4 mm colonies after 24 hours' incubation at 37°C (MAFF 1984, Oxoid Manual 1982).

Triple Sugar Iron (TSI) Agar: This medium contains lactose, sucrose and dextrose and an indicator for the detection of hydrogen sulphide. The phenol red in this medium becomes yellow in acid conditions and red in alkaline conditions (Williams et al. 1980).

Salmonella does not ferment lactose and sucrose but ferments the dextrose (Edwards and Ewing 1962) and produces gas and acid reactions in the butt of the TSI slope. This acid on the slant surface changes to alkaline end products by oxidation. Hydrogen sulphide changes the colour of the butt to black (Williams et al. 1980).

Urea Broth: Bacteria such as Proteus and some citrobacter species are able to produce the same reaction as Salmonella on TSI (MAFF 1984). They can be differentiated from Salmonella by their ability to produce urease and hydrolyse the urea of urea broth medium. This hydrolysis changes the pH of the medium from acid to alkaline and produce pink colour in the media. Salmonellae do not produce urease and are negative by this test (Edwards and Ewing 1962, MAFF 1984, Williams et al. 1980, Oxoid Manual 1982).

C) Pathogenicity

Salmonella organisms of the paratyphoid group can infect birds of all ages but mortality is greatest in young birds with a peak at 6-10 days of life (Williams 1984). Seuna (1979)

showed that young chicks are very sensitive to S. typhimurium infection and that in many cases a single organism could infect chicks. He also showed that a dose of 15 S. typhimurium organisms per chick was able to produce 100% infection in flocks when given to day old chicks.

Mortality in young birds in natural conditions varies from very low up to about 20% while mortalities of 80% or more have been recorded in severe outbreaks (Williams 1984). Barrow et al. (1987a) reported 0 to 100 percent mortality following the oral inoculation of 10 different strains of S. typhimurium in Light Sussex chicks during the first three weeks of life.

Adult birds are more resistant to infection but infection can occur especially if they are stressed before exposure (Whiteman and Bickford, 1983). Lee et al. (1983) found that infection with S. typhimurium results in colonisation of the gut in adult birds and infected birds remain carriers for at least 98 days. After initial infection of the intestinal tract, organisms can cause systemic infection. The organism can be isolated from the liver, spleen and bursa for at least up to 42 days after exposure. Organisms may be isolated from both small and large intestines and the caecal tonsil after day 6 of exposure and persist in the last site up to 98 days postexposure (Lee et al. 1981 and 1983). Adult birds do not show any clinical signs (Williams 1984) and remain asymptomatic carriers for several weeks. These carriers are the most dangerous and potential source of infection to the flock (Lee et al. 1983, Clarke and Gyles 1986).

D) Pathogenesis

Infection occurs by a number of routes and is then followed by multiplication of the organism in a number of sites. Once infection has occurred, two major factors determine the outcome of the infection. One is the pathogenicity of the organism and its ability to challenge the immune system of the

host. The other is the host response and its ability to combat the organism and this is reviewed in Section 3 below.

a) Routes and sources of infection

Salmonella can enter the body via the respiratory tract, pharynx, conjunctiva and, most commonly by the oral route (Clarke and Gyles 1986). The oral and percloacal routes were considered to be the commonest portals of entry in natural infection (Lee et al. 1983) and these routes were used by Brown et al. (1975) and Leaney et al. (1978) to establish gut colonisation and systemic infection using S. typhimurium in the chicken. Lee et al. (1983) showed that injection of S. typhimurium into the jugular vein of 8 week old chickens can lead to systemic infection, gut colonisation and shedding of the organism in the faeces. Contaminated faeces can infect other chickens in the flock by contamination of water and feed and can also contaminate egg shells in layer flocks and in turn represent a potential source of infection in hatcheries. Egg shells can be contaminated by passage through the cloaca of an infected hen or later on by contact with contaminated faeces, floors, nests etc. Salmonella is then able to penetrate the egg and multiply within it. There is little evidence to support direct ovarian transmission of paratyphoid Salmonella into eggs as a common route of infection (Williams 1984). Forsythe et al. (1967) could not establish this pattern except for S. pullorum. Following oral inoculation of hens with high doses of S. typhimurium, S. senftenberg and S. thompson, there was no Salmonella isolation from eggs, ovary and internal organs but the organism was recovered from egg shell surfaces and faeces indicating that direct ovarian transmission of these organisms was unlikely (Cox et al. 1973). However, recent epidemiological evidence suggests that S. enteritidis can be transmitted by this route and it has been isolated from eggs and the ovaries of birds at post mortem (Hopper and Mawer 1988, Lister 1988).

When Salmonella get into a hatchery via eggs or other

means, they are easily distributed among the chicks by contact or in the air. Fluff and dust often lead to high levels of infection among all chicks in that hatchery (Williams 1984).

All species of birds, mammals including human, fish, reptiles and insects are reported to be carriers of Salmonella and can transmit the infection to the chicken. Feeds especially those of animal origin such as fish meal, bone meal and meat meal are also potential sources of infection (Clarke and Gyles 1986, Williams 1984) and in Britain levels of 66.7%, 42.9%, 26.9%, 37.9%, 13.6% and 4.5% infection have been reported in bone meal, feather meal, meat and bone meal, poultry offal meal, blood meal and white fish meal respectively during the period of 1982 to 1986 (Anon. 1987).

b) Colonisation and invasion

Salmonella organisms are able to colonise the whole intestine of the chicken (Lee et al. 1983). Soerjadi et al. (1982) studied the attachment of S. typhimurium to the intestinal epithelial surface of germ free chicks and showed that they adhered firmly to the epithelium of the caecum and, in the absence of other microflora, were able to colonise all parts of the gastrointestinal tract from oesophagus to cloaca. In the presence of the normal flora of the gut, Salmonella colonise the crop and caeca in greatest numbers. This phenomenon is discussed below under the topic of non specific defence mechanisms of chickens. Attachment may be due to type 1 fimbriae (Klemm 1979, Jones et al. 1981) or non fimbrial attractive forces (Jones et al. 1981).

After adherence to the epithelial wall the Salmonellae enter the epithelial cells by a process similar to endocytosis and then migrate to lamina propria through the extra cellular spaces in macrophages (Popiel and Turnbull 1985). Their study supports the work of Takeuchi and Sprinz (1967) who found that bacteria enter the cells through the

microvilli or through the junctional complexes between the enterocytes enclosed in a membrane-bound vacuole that migrates to the basal region of the cell. In the lamina propria the bacteria stimulate an inflammatory response and are engulfed by macrophages and neutrophils (Clarke and Gyles, 1986). Salmonellae multiply inside the phagocytes (Clarke and Gyles, 1986) and cause death of the cells and are released back into the lumen of the gut (Popiel and Turnbull 1985).

Clarke and Gyles (1986) stated that Salmonella infection in poultry results in shortening of the villi, degeneration of enterocytes, depletion of goblet cells, a neutrophilic reaction in the lamina propria and inflammation and fluid secretion in the intestine. Inflammation and intestinal fluid secretion was reported by Gianella et al. (1973) using rabbit ileal-loop as a model and various strains of S. typhimurium.. They suggested that intestinal fluid loss might be due to mucosal invasion of Salmonella and a bacterial factor such as enterotoxin might be responsible for fluid secretion. Using the same model and organism, Wallis et al. (1986b) showed that fluid secretion can occur without any gross architectural damage and supported the idea that Salmonella-induced diarrhoea is a secretory diarrhoea.

In acute enteritis due to loss of fluid and electrolytes, death may occur without systemic invasion (Clarke and Gyles 1986). If the animal survives local infection, the Salmonellae can get into the circulation via the lymph nodes and efferent lymph vessels and become distributed throughout the body. After systemic involvement, the reticuloendothelial system filters out the organism. Some organisms are able to multiply in the body and localise in organs such as the gall bladder and ovary. It is also postulated that endotoxin might be responsible for fever, thrombosis and other systemic effects of the disease (Clarke and Gyles 1986). In this case, if the host animal can not overcome the disease the outcome will be septicaemia and the development of pathological lesions in different organs of

the body such as pericarditis, perihepatitis, necrotic foci in the heart and liver, airsacculitis, ocular lesions, meningitis, conjunctivitis and arthritis in birds (Williams 1984).

c) Virulence factors of Salmonella

Knowledge of factors that contribute toward the virulence of micro organisms *in vivo* give us a better insight into the mechanism of pathogenicity of this organism and is an important starting point for the control and prevention of salmonellosis (Parry and Porter 1981). These factors may be bacterial structures that help the Salmonella to attach to the mucosa and multiply and produce disease (Woolcock 1979, Lindberg 1980) or their products. Some of the known factors are reviewed follow:

(i) Enterotoxins

S. typhimurium is able to produce a toxin similar to cholera enterotoxin (Woolcock 1979, Clarke and Gyles 1986) and immunisation against cholera toxin protects against the effect of live S. typhimurium in intestinal loops (Woolcock 1979). The enterotoxin of S. typhimurium is heat-labile and can cause fluid accumulation in rabbit intestine. The enterotoxins of cholera and E-coli do not penetrate into epithelial cells and it is suggested that they bind to a receptor on surface membranes of microvilli, stimulate the production of adenyl cyclase and, in association with this enzyme, cause intestinal hypersecretion (Woolcock 1979). Wallis et al. (1986a) examined six strains of Salmonella typhimurium for toxin production *in vitro* and studied the effect of the toxin in relation to fluid secretion in rabbit ileum. They showed that invasion by bacteria is required for fluid secretion by enterotoxin and that there is no correlation between *in vitro* enterotoxin production and the ability of whole organisms to produce fluid secretion. Enterotoxin extracted from all six strains *in vitro* failed to show any activity in cell culture and infant mouse

assay tests.

(ii) Cytotoxin

Koo et al. (1984) studied the effect of Salmonella cell lysates on Vero cells and isolated epithelial cells of rabbit intestine and showed that the heat-labile cytotoxin of Salmonella is able to inhibit protein synthesis in these cells and consequently to alter the cell membrane. Similar results obtained with in vivo studies on rabbit intestinal loops suggest that Salmonella cytotoxin might be responsible for the damage and sloughing of the epithelial cells during salmonellosis. Entry of and spread of the organism into the intestinal tissue may be enhanced by this damage. The mechanism of release of this cytotoxin and whether or not it plays a part in pathogenesis during systemic infection is unknown.

(iii) Endotoxin

Endotoxin is produced by all Enterobacteriaceae. There is a close relationship between O antigenicity and endotoxic properties, but the structures responsible for O specificity can be distinguished from those responsible for endotoxic activity (Luderitz et al. 1966).

Lipopolysaccharide (LPS) is a building block of outer membranes of all Salmonella organisms. The polysaccharide part helps the organism to resist the host defences and the lipid part (called lipid A) is responsible for tissue damage and endotoxic activity (Lindberg 1980).

In general the toxic activities of lipid A are pyrogenicity, bone-marrow necrosis, leucopaenia, leucocytosis, hypotension, abortion, Schwartzman phenomenon, tumour necrosis, lethality (Lindberg 1980) and thrombosis and vascular damage in the intestinal mucosa (Clarke and Gyles 1986).

The outcome of Gram-negative bacteraemia can be prevented by antibodies directed against lipid A. These antibodies have antitoxic effects rather than opsonic or bactericidal in serum resistant strains. There is some opsonic ability in susceptible strains. For antibodies to have this effect, the lipid A must be exposed sufficiently and in smooth strains it is difficult for the antibodies to react with it. It is important to know that the antigenic sites on lipid A are cross reactive among all Enterobacteriaceae and in theory there should be cross protection by antibodies directed against lipid A. In practice this cross protection does not occur because of the hindrance of lipid A (Woolcock 1979).

Some activities of lipid A such as pyrogenicity, the induction of non-specific resistance to infection and enhancement of the immune response are beneficial to the host (Woolcock 1979, Lindberg 1980). The mechanism of these biological effects is not obvious (Lindberg 1980) and the role of endotoxin in individual disease states is not well established (Woolcock 1979).

(iv) Lipopolysaccharide

According to Woolcock (1979) and Lindberg (1980) the LPS of Salmonella is situated on the external part of the bacterial cell wall and is composed of the following layers:

a) Surface polysaccharide that is in the form of repeating polymerised oligosaccharide units. This structure is the basis of O antigenic specificity in different serogroups.

b) The core or basal oligosaccharide chain that is common to all Salmonella organisms.

c) The lipid A portion of LPS that is attached to the bacterial cell wall protein layer.

These three layers are covalently linked together (Lindberg 1980). The first layer or O polysaccharide chain is not present in the rough mutants of Salmonella and other Enterobacteriaceae (Woolcock 1979, Lindberg 1980). Rough mutant of Salmonella with deficiencies in the LPS of their cell walls are of substantially lower virulence (Nakano and Satio 1969, Morris et al. 1976, Woolcock 1979, Lindberg 1980, Clarke and Gyles 1986). Nakano and Satio (1969) demonstrated the role of different components of S. typhimurium cell wall in virulence and immunogenicity in mice. They studied a series of different mutant strains of this organism by intraperitoneal injection into mice and measured their virulence, immunogenicity and protective effect by subsequent infection with wild type (smooth) strains. The strains with complete O side chain were most virulent. Lack of part(s) of this chain rendered the organism less virulent and the bacteria with the exposed core were not virulent at all. The multiplication of these organisms in the bodies of mice was dependent on the completeness of the O polysaccharide chain and, as the sugar components were lost in different mutant strains, their ability to multiply was reduced. Organisms with LPS containing one heptose plus 3-deoxyoctulosonate (KDO) and KDO alone were not able to multiply. The mutants that were able to multiply in the host induced protective immunity against the wild type of Salmonella (smooth strain) in subsequent challenge. As a result they suggested that rough mutants of S. typhimurium might be suitable for vaccination and concluded that antibodies against the O chain are not essential for protection.

Morris et al. (1976) showed that galactose-4-epimerase (gal-E) mutant strains of S. typhimurium are unable to multiply in the liver and spleen of CBA mice. Gal-E mutant strains have a defective uridine diphosphate (UDP) galactose-4-epimerase enzyme that makes them unable to synthesise UDP galactose from UDP glucose. Consequently they cannot incorporate this sugar into the LPS and appear rough rather than smooth (Germanier 1970).

Germanier (1970) reported that loss of the O-specific side chain or a decrease in the qualitative contents of cell wall LPS causes a sharp decrease in virulence of S. typhimurium. Loss of O specific side chains resulted in a 10,000 fold decrease in virulence, while further degradation of LPS caused only slight reduction in virulence. This view was supported by Woolcock (1979) and Lindberg (1980).

Several different factors might be responsible for this reduction of the virulence of rough mutants e.g. more sensitivity to fatty acids in the intestine (Lindberg 1980), less protection against lysosomal attack, more susceptibility to the bactericidal effects of granules, less protection against antigen-antibody mediated reactions (Clarke and Gyles 1984).

Thus the presence of the O polysaccharide chain is very important in resistance to host defences. The qualitative changes of the O polysaccharide chain in different isogenic species also affects the virulence. For example, the O chain of S. typhimurium has slight differences in the nature of the dideoxyhexosyl substituent of the D-mannosyl residues when compared with that of S. enteritidis. Replacing the O side chain of S. typhimurium with that of S. enteritidis makes it 10 fold less virulent (Lindberg 1980).

(v) Flagella

Flagella are filamentous appendages on the surface of many bacteria that enable them to swim actively by rotating like propellers. They are composed of three parts a) a distal filament, b) a short hook and c) the proximal complex basal structure (Doetsch and Sjoblad 1980, Rogers 1983).

The filament is made of a single subunit of protein called flagellin with a molecular weight of 51000-57000 daltons in Salmonella species (Doetsch and Sjoblad 1980). The amino acid

composition of flagellin of different species is different, but all lack cysteine and tryptophan. N-methyl lysine is present only in Salmonella flagellin (Doetsch and Sjoblad 1980, Roger 1983). Brief exposure to acid, alkali, alcohol, formaldehyde, freezing and thawing or ultrasonication breaks up the filaments into fine wavy fibres (Rogers 1983).

The hook connects filaments to the basal structure and consists of a single protein with molecular weight of 42000 daltons in E. coli or Salmonella (Rogers 1983). The protein of the hook is antigenically distinct from flagellin (Doetsch and Sjoblad 1980) and antibodies against this protein do not cross react with flagellin (Rogers 1983).

The basal structure of flagella is very complicated and composed of one rod and four rings or discs in E. coli and Salmonella organisms. These rings are associated with LPS, the peptidoglycan layer and the cytoplasmic membrane of the bacteria (Doetsch and Sjoblad 1980). The basal structure is made up of 9-13 proteins (Rogers 1983).

Flagella can be sheared from bacteria by blending (Parton 1975, Rogers 1983) and the growth of Salmonella at 44^{o}C inhibits the production of flagella (Parton 1975).The role of flagella in the development of disease is not yet obvious. Doetsch and Sjoblad (1980) suggested that the presence of different charge polarities in regions of filaments may play a part in flagellar attachment to surfaces and Weinstein et al. (1984) showed that after the oral inoculation of mice with flagellated and non flagellated S. typhimurium the former were able to grow faster in the liver and spleen and that after intravenous challenge the flagellated organisms multiplied logarithmically until the mice died but the non flagellated organisms increased only slightly and did not kill the mice. They concluded that flagella protect the organism in one of these two ways, a) by protection of the organism against intracellular killing by murine macrophages. b) Increasing the

ability of the organism to multiply inside the macrophages. Barrow and Lovell (1989) supported this contention by reporting that motile strains of S. typhimurium and S. infantis are more invasive than non motile strains.

(vi) Fimbriae

Fimbriae or pili are thread-like proteinaceous appendages, 0.5-1.5 um long and about 7 nm in diameter, found on the surface of many bacteria (Klemm 1979). It is known that fimbriae are the means by which pathogenic bacteria adhere to the epithelial cells of the host. In E. coli these fimbriae are known as colonisation factor antigens (CFAs) I and II in human (Evans et al. 1984, Gyles 1986), K88 antigen in pigs and K99 antigen in cow and sheep, and that many are plasmid mediated (Klemm 1979, Faris et al. 1984, Gyles 1986). All of the pili mentioned are D-mannose resistant (Gyles 1986) and low concentration of mannose can not impair their adherence to animal cell surfaces.

Apart from these types of pili there is another type that is sensitive to D-mannose and can haemagglutinate the red blood cells of man and guinea pigs called type 1 pili (Gyles 1986, Sharon 1984). The type 1 pili are present on the surface of E. coli (Gyles 1986) and S. typhimurium (Faris et al. 1984, Sharon 1984). Sharon (1984) reported that the presence of lectins on type 1 pili play a key role in bacterial adherence to D-mannose on animal cells and treating the cells with sodium metaperiodate (oxidises sugar residues on cell surface) or concanavalin A (specifically binds to mannose or glucose residues on cells) prevented the attachment of the bacteria. Gyles (1986) could find no conclusive evidence for the implication of type 1 pili as virulence factors and Barrow and Lovell (1989) showed that type 1 pili might not be involved in invasion by Salmonella species and stated that D(+)mannose did not reduce the invasion of Vero cells by 11 strains of Salmonella.

(vii) Plasmids

Jones et al. (1982) reported the presence of a 60 megadalton plasmid in six invasive strains of S. typhimurium and showed that elimination of these plasmids significantly decreased their adhesive capacity and rendered them unable to invade HeLa cells. Also two strains tested on mice by oral inoculation were either avirulent or less virulent in comparison to the parental untreated strains.

Clarke and Gyles (1986) concluded on the basis of Popoff's work in 1984 which they cited, that, due to the isolation of plasmid free Salmonella from outbreaks of salmonellosis, the virulence plasmid is not essential for pathogenicity in Salmonella. However Barrow and Lovell (1988) proved that an 85 kilobase plasmid in S. pullorum was responsible for virulence and high mortality in Rhode Island Red chicks when given by the oral route. There was no mortality and morbidity after oral inoculation of plasmid cured strains while virulence of the organism was restored after reintroduction of the plasmid.

The large plasmid of S. gallinarum is a major virulence factor, without it the bacteria cannot survive and grow in the cells of the reticuloendothelial system and plasmid free organisms are unable to invade the epithelial cells of the alimentary tract and enter the circulation (Barrow et al. 1987b). There was no relation between the cryptic plasmid of S. typhimurium and colonisation of Peyer's patches in mice and invasion of HeLa cells (Hackett et al. 1986). The latter finding was supported by Barrow and Lovell (1989) who also demonstrated that virulence-associated plasmids were not required for the invasion of Vero cells by S. typhimurium, S. gallinarum and S. pullorum.

3 - PROTECTION AGAINST AND IMMUNITY TO SALMONELLA IN THE CHICKEN

Despite the enormous amount of work that has been carried out on avian salmonellosis , the exact mechanism of immunity and the role played by different parts of avian immune system in defence against Salmonella is not obvious. Immunological aspects of protection against S. typhimurium and the role of different antibodies and cell mediated immunity are not yet established.

The subject is reviewed here with special emphasis on the avian immune system and the factors affecting Salmonella infection in chickens. In appropriate places references are made to S. typhimurium.

A) The avian immune system and specific immunity

The avian immune system is composed of the bursa of Fabricius (the differentiation site of B-cells that produce immunoglobulins), the thymus (responsible for T cell production and cell mediated immunity), the spleen, the caecal tonsils, the mural lymph nodules, the lymph nodules, the pineal gland, the Harderian glands and the Peyer's patches. These provide immunity to the bird in a similar manner to their equivalent in mammals. There are, however, some special characteristics that distinguish the avian from the mammalian immune system.

a) The bursa of Fabricius is a lymphoepithelial organ exclusive to birds (Firth 1977, King and McLelland 1984).

b) Functionally and anatomically the avian immune system can be divided into two distinct parts. From a functional point of view it is composed of bursa dependent components responsible for humoral immunity and a thymus dependent component that is responsible for cellular immunity. From an anatomical point of view, the bursa and thymus constitute the central part of the

immune system and the other lymphoid tissues listed above are components of the peripheral lymphoid system (Firth 1977, Glick 1986).

c) Avian lymph nodules are different from those in mammals and there is no filtering role (Firth 1977) or the nodules are less efficient in filtration than their mammalian counterparts (King and McLelland 1984). There is no true lymph node in the domestic fowl.

d) The immunoglobulins of the fowl are not similar in their physical and chemical properties to their equivalents in mammals (Higgins 1975).

a) Central lymphoid tissues

(i) Bursa of Fabricius

The bursa is a hollow round or oval sac in the dorsal region of the proctodeum of the cloaca (White 1981, Glick 1986). Its shape varies in different species of birds (King and McLelland 1984, Glick 1986). It becomes fully developed in immature birds (Wright 1981) and begins to involute at 2-3 months of age and involution becomes advanced at the time of sexual maturity (King and McLelland 1984). The first sign of bursal development appears between days 3 and 5 of incubation (Firth 1977, Glick 1986). Lymphoblastic activities can be seen around day 13 (White 1981) and 14 of incubation (Firth 1977). It is believed these lymphoblasts migrate from elsewhere, and are most probably stem cells of yolk sac origin (Toivanen et al. 1981, White 1981, King and McLelland 1984) and enter the bursa on day 8 of incubation and multiply there by mitosis (Toivanen et al. 1981).

The medullary parts of bursal follicles are formed by the proliferation of lymphocytes during incubation and the cortical parts develop after hatching (Toivanen et al. 1981). The

follicles form distinct structures in the developed bursa (White 1981) and there are about 8000-12000 bursal follicles in each bursa (Glick 1986). The follicular medulla is composed of lymphocytes, lymphoblasts, reticulocytes, macrophages, a few plasma cells and secretory cells and the follicullar cortex has lymphocytes, lymphoblasts, macrophages and plasma cells (Glick 1986). Follicle associated epithelial cells cover the bursal follicles and by their pinocytic activity take a variety of soluble substances from the gut lumen into the medulla of the bursal follicles. Introduction of antigen in this way leads to antibody production (Glick 1986, Toivanen et al.1987) that is supposed to be important in gut local immunity (Firth 1977).

The microenvironment of the bursa is where differentiation occurs of the immunologically competent bursal (B) lymphocytes that are responsible for avian antibody production (Ratcliffe et al. 1987). In this site the stem cells change to B-lymphocytes, expression of the Specific V region gene takes place and multiplication within the follicles and development of the capacity to migrate to the periphery occurs (Boyd et al. 1987). This process happens between the 8th day of incubation and hatching time. Around the 18th day of incubation there are no more stem cells in the periphery and mature B-cells migrate to peripheral organs and the circulation. The B-cells are capable of replication and in this way compensate for the restricted stage of B-cell development in the bursa, in contrast to the mammalian system where there is a constant production of B-cells from bone marrow during life (Ratcliffe et al. 1987).

Most of investigators have demonstrated the above roles for the bursa, but some have claimed that the bursa might not be necessary for the development of the B-cells because they can be seen after bursectomy or suppression of the bursa in embryos (Firth 1977). Apart from these immunological functions, the bursa may have hormonal functions as well. After bursectomy the response of the chicken to ACTH and adrenalin is diminished

and the uptake of iodine by the thyroid gland is also influenced (Firth 1977).

(ii) Thymus

The avian thymus consists of seven lobes on each side of the neck (White 1981, Glick 1986). The number of the lobes may vary from three to eight pairs (King and McLelland 1984). They originate from the third and fourth pharyngeal pouches of the embryo (Firth 1977, White 1981, King and McLelland 1984) and they are the first lymphoid structure that appears in the embryo (Firth 1977). At hatching time it comprises 0.3% of the body weight (Firth 1977) and reach as its maximum size between 4 and 14 weeks of age in domestic fowl and then begins to involute (King and McLelland 1984).

The histological structure of fowl thymus is similar to that of mammals (White 1981, King and McLelland 1984). The structural units of the thymus are lobules that comprise the thymic lobes. Each lobule consists of two parts, medulla and cortex. Both contain reticular cells, fibres and small lymphocytes. The latter cells are fewer in the medulla than in the cortex (Firth 1977, King and McLelland 1984). There is a blood-thymus barrier only in the cortex and its role is thought to be the protection of the cortical thymus from exposure to antigens. It is known that anti-thymocyte sera do not affect the cortex while destroying the recirculatory thymic (T) cells in the circulation (White 1981).

T-cells or thymic lymphocytes are derived from circulatory stem cells that originated from the yolk sac which during embryonic life migrated to the thymus, developed and differentiated there (King and McLelland 1984). The pro-thymic cells may also originate from bone marrow and can be seen after hatching (Ratcliffe et al. 1987). Large lymphocytes (11 um) appear in the thymus on day 11 of incubation and their size decreases as the embryo ages until they are half the original

size (5.5 um) on day 16 of incubation (Glick 1986). The thymus also contains some B-cells (King and McLelland 1984).

The thymic derived cells consist of three subpopulations: a) T-helper cells that help the immunoglobulin synthesis, b) T-suppressor cells that modulate a variety of humoral and cellular responses, and c) T-cytotoxic cells that help the body defence mechanism against intracellular pathogens by their cytotoxic effect on the cells harbouring the pathogens. T-cells are responsible for cell-mediated immunity (Glick 1986).

b) Peripheral lymphoid tissues

(i) Spleen

The spleen of the domestic fowl is reddish brown, spherical organ, 2 cm in diameter and located on the right side of the junction between proventriculus and gizzard (King and McLelland 1984). This organ contains both T-cells and B-cells (Firth 1977, King and McLelland 1984) in both red and white pulp. The functions of spleen are:

a) Erythrocyte destruction by macrophages through phagocytosis in the red pulp.

b) Production of lymphocytes in the white pulp.

c) Production of antibody by B-cells and T-helper cells in the red and white pulp (Firth 1977, King and McLelland 1984).

Circulation brings the antigens to the spleen and consequently the spleen is the main source of antibodies against those antigens that are found in the blood stream (White 1981, Eerola et al. 1987). Germinal centres are found in the islands of white pulp and their number increases after chronic infection (White 1981) and immunisation (Eerola et al. 1987). The germinal centres are composed of B-cells but T-cells

are required for their formation (Eerola et al. 1987).

Twenty four hours after the entrance of antigen or an infectious agent into the circulation the plasma cells in the spleen will produce antibody and this early produced antibody may help to localise the antigen in the white pulp as antigen-antibody complexes. The number of plasma cells reaches a maximum after one week and the peak antibody level is produced 8-9 days post infection. Around two weeks after infection, the antibody forming cells of the newly formed germinal centres produce antibody and persist for up to six weeks. It is thought that the germinal centres may act as memory cells and induce further antibody production after reintroduction of the antigen or infectious agent (Firth 1977).

(ii) Lymph nodes

The lymph nodes are not present in domestic fowl and only ducks and some other aquatic species have lymph nodes similar to those of mammals (White 1981, King and McLelland 1984, Eerola et al. 1987).

(iii) Mural lymphoid nodules

These are small collections of lymphoid tissues without a capsule or well defined boundary located alongside the lymphatic vessels in fowl and some other avian species (White 1981, King and McLelland 1984). Each nodule has a few germinal centres that appear around six week after hatching (White 1981). These nodules have little or no filtering role (Firth 1977, White 1981).

(iv) Caecal tonsils

The caecal tonsils are a pair of aggregated lymphoid nodules (King and McLelland 1984) in the proximal wall of each caecum (Glick 1986) near its junction with ileum (White 1981).

They are located in the lamina propria and submucosa and are composed of germinal centres that are separated by connective tissue containing mature and immature plasma cells. The caecal tonsils are fully developed about five weeks after hatching and germinal centres grow more rapidly and bigger in size in comparison to those of the spleen (White 1981). The caecal tonsils have both B and T-cells (White 1981, Glick 1986) and they are an important source of antibody. IgA, IgM and IgG biosynthesis have been reported in the caecal tonsils (White 1981).

(v) Peyer's patches

They are found in the wall of the ileum of intestine of the domestic fowl (White 1981). Most investigators regard them as similar to caecal tonsils (Glick 1986) but the germinal centres are not well developed (White 1981) and they present an abnormal response to some antigens (King and McLelland 1984).

(vi) Harderian and pineal glands

The Harderian glands are paraocular glands and contain large numbers of plasma cells that are responsible for IgA production which is important in local immunity (White 1981). Glick (1986) reported the presence of monomeric IgM on the membrane of the B-cells of this gland. He also demonstrated the presence of B and T-cells and antibody production in the pineal gland.

c) Humoral immunity

The components of the chicken immune system and the role of each one in immunity has been described above. The bursa dependent lymphocytes are considered responsible for antibody production but it is realised that in most cases effective immunity develops only as the result of interaction between both B and T-cells and also some other cells and factors that

make the complexity of immunological reactions. The rest of this chapter is devoted to a review of the immunoglobulins as the product of humoral immunity, their role in the defence against Salmonella infection (both active and passive immunity) and also the role of cell mediated immunity. Salmonella vaccines are also reviewed.

(i) Immunoglobulin synthesis

The immunoglobulin (Ig) classes in the chicken are IgA, IgM, IgG, IgE (Ivanyi 1981, Glick 1986) and IgD (Rose and Orlans 1981, Glick 1986, Benedict and Berestecky 1987). Immunoglobulins are glycoprotein in nature and synthesised by the B-cells and plasma cells. Each antibody is composed of two identical heavy (H) and two identical light (L) chains that are held together by disulphide bonds (Glick 1986). Upon digestion by enzymes an antibody molecule divides into two parts, the "fragment crystalline" (Fc) portion that will crystallise after purification and the "fragment antigen binding" (Fab) portion that binds specifically to antigen (Bourne 1983). The Fab part of different immunoglobulin isotypes may show similar affinity toward an antigenic determinant. The antibodies can then be distinguished from each other by the differences in the constant (Fc) parts of their heavy chains (Glick 1986).

Leslie and Clem (1969) found that antibodies against the L chain of one immunoglobulin class are cross reactive with the L chains of another class. The antibody is produced in response to exposure to an antigen. After the first exposure of chickens to an antigen, a primary response will occur that consists of a latent phase (the activation time for immunocompetent cells), an exponential phase in which antibody increases sharply to a certain level (peak) and declining or regression phase in which the antibody level declines. Reexposure of the bird to the same antigen causes a secondary response with a shorter latent period, a greater exponential phase slope, higher and earlier peaks and longer lasting antibody titre. The antibodies of the

primary response are mainly IgM and those of the secondary response mainly IgG (Glick 1986). According to Parry and Porter (1981) in Salmonella infected chickens these responses are influenced by the age of the bird and the serotype of the organism. They reported that S. pullorum and S. gallinarum stimulate higher titres of IgM antibody production than the paratyphoid group. The best response is obtained with live bacteria at the age of 22-25 weeks of age, the time of immunological maturity.

Antigens stimulate antibody production either by direct attachment to the B-cells and their activation to plasma cells that proliferate and produce specific immunoglobulins against that antigen (Roitt 1988) or bind to macrophages that lead to the activation of T-cells and then B-cells (Glick 1986). The function and characteristics of the fowl immunoglobulins and their distribution in the body fluids and secretions are reviewed below.

(ii) IgA

The presence of an immunoglobulin similar to mammalian IgA was first reported by Lebacq-Verheyden et al. (1972a) and Orlans and Rose (1972). It is regarded as a secretory immunoglobulin (Leslie and Martin 1973) but is not identical to its mammalian counterpart (Higgins 1975) and is antigenically distinct from human IgA (Sanders and Case 1977).

IgA is present in fowl serum (Lebacq-Verheyden et al. 1972a and b, Orlans and Rose 1972, Leslie and Martin 1973) in a concentration of 0.33 mg/ml (Lebacq-Verheyden et al. 1974). It is the major immunoglobulin in chicken bile and intestinal secretions (Lebacq-Verheyden et al. 1974, Watanabe and Kobayashi 1974) and is also present in tear fluid, saliva, oviductal mucus, seminal plasma, urine (Watanabe et al. 1975), tracheal washing (Chhabra and Goel 1980) and egg white (Rose et al. 1974, Goudswaard et al. 1977a and b, Rose and Orlans 1981).

The molecular weight of immunoglobulins in serum and secretions is shown in Table 1.

As described above, the presence of IgA in the serum and secretions of avian species is well established and it is the main secretory immunoglobulin found at the mucosal surfaces (Lim and Maheswaran 1977). The presence of secretory component (SC) in chickens has been reported from bile IgA (Leslie and Martin 1973, Porter and Parry 1976, Parry and Porter 1978, Rose et al. 1981), egg white (Rose et al. 1974) and intestinal secretions (Watanabe and Kobayashi 1974).

There is no cross reaction between the heavy chain of IgA and the H chain of IgG and IgM (Leslie and Martin 1973, Sanders and Case 1977). Porter and Parry (1976) showed that there is no cross reaction between chicken and mammalian IgA using haemagglutination inhibition assays.

Although the presence of cells with membrane bound IgA has been reported in the yolk sac at day 5 and cells containing IgA in the bursa from day 11 of incubation, serum IgA is not detectable until 10-12 days after hatching. In gall bladder, secretory IgA is found at day 8-11 post hatching (Rose and Orlans 1981).

IgA is mainly produced by plasma cells which have originated from the bursa of Fabricius (Toivanen et al. 1987) and are present in the lamina propria of the gut of both mammalian and avian species (Lee et al. 1981). In the chicken the plasma cells are found in the trachea (Riddell 1987), caecal tonsils, Peyer's patches (White 1981), lung, spleen, thymus, bone marrow, blood (Lawrence et al. 1979) and beneath the epithelium of the bronchi and the oviduct (Lebacq-Verheyden et al. 1972b). The latter authors found that IgA containing cells are predominant among the other immunoglobulin containing cells in the lamina propria of the mucosa of duodenum, jejunum, ileum, caecal tonsils and caecum. The work of Bienenstock et

Table 1

The molecular weight of chicken immunoglobulins in the serum and secretions (K daltons).

	IgA	IgM	IgG	IgD
Monomer or polymer	170 & 340 (S, 1) 170-200 (S, 2) 350 & 900 (B, 2) 350 & 500 (I, 2) 170 (S, 3) 350-360 (B, 3) 300 (I, 4) 650 (T & Sa, 5) 710(B, OM & U,5) 350 (I, 5)	184 & 920 (S, 1) 880-890 (S, 2)	165-180 (S, 2)	
H chain	70 (3) 60 (4) 76 (6)	62.6-70 (2) 70 (3) 79 (6)	60.5-68 (2) 70 (6)	81 (6)
L chain	24 (4) 25 (6)	22-23.9 (2) 25 (6)	22-23 (2) 25 (6)	25 (6)

The letters inside the parentheses indicate the origin of the immunoglobulin and numbers represent the authors as described below.

B = Bile I = Intestine OM = Oviductal Mucus S = Serum
Sa = Saliva T = Tear U = Urine

1 - Schranner and Losch (1986).
2 - Benedict and Berestecky (1987).
3 - Leslie and Martin (1973).
4 - Sanders and Case (1977).
5 - Watanabe et al. (1975).
6 - Chen and Cooper (1987).

al. (1973) supported this view and they could not find IgA cells in the crop and Peyer's patches, but reported the presence of these cells in the spleen.

The IgA that is found in the exocrine secretions of the intestine, reaches them by two ways (Jackson et al. 1978).

a) As a result of an immune response to local or systemic infection or antigen introduction, the precursor B-lymphocytes in the intestinal wall are stimulated through the action of T-helper cells and become IgA precursor lymphoblasts that migrate via the lymphatic vessels of the intestine to the circulation and home to the lamina propria of the gut and other places that are involved in IgA production (Stokes 1984) such as respiratory, intestinal, genital and urinary tract, oviduct and interstitial tissues of exocrine glands e.g. the lacrimal gland and the salivary gland. These cells produce IgA antibody that passes through the columnar epithelial cells (Jackson et al. 1978, Lee et al. 1981) by the process of pinocytosis and reverse pinocytosis after binding to the secretory components that are synthesised on the surface of the cell membrane of the epithelial cells (Parry and Porter 1981, Genco et al. 1983).

b) The other way is by active transportation through the bile. The IgA that is synthesised by plasma cells is passively transported through the mesenteric and thoracic lymph ducts to the general circulation where it is filtered out into the bile selectively by hepatic cells (Jackson et al. 1978). These studies were carried out on the rat, but Rose et al. (1981) confirmed the biliary route of transportation for IgA in the chicken. They showed that radiolabelled monoclonal dimeric and monomeric human IgA injected into the wing vein were secreted in the bile. Ligation of the two hepatic bile ducts resulted in a 3-4 fold increase in concentration of IgA in the serum. Due to the active removal of IgA from serum by the liver, the concentration of this immunoglobulin in the serum remains very low (Jackson et al. 1978, Rose et al. 1981).

The presence of IgA on the mucosal surface of the oviduct (Watanabe et al. 1975), in oviductal secretions (Orlans and Rose 1972) and in egg white (Rose et al. 1974) of the chicken is reported but the exact mechanism of secretion is not yet established. Dohms (1978) did not find a significant amount of radiolabelled IgA to be transferred from turkey serum into the egg. As there is 0.7 mg of IgA in 1 ml of chicken egg white (Rose et al. 1974) and there are IgA containing cells in the epithelium of the oviduct, it is probable that these immunoglobulins are produced locally (Rose et al. 1981) and enter the egg white while it is secreted around the yolk and passes down through the oviduct.

Ewert et al. (1979) showed that vaccination of the chicken with Newcastle disease virus either locally or parenterally will produce IgA in serum and secretions. They showed also the presence of IgA and IgM specific to this virus in the egg white. The importance of IgA and its role as the first line of defence on mucosal surface against viruses is well established but there is no conclusive evidence about its role against bacterial pathogens in poultry. It has been shown that the development of local defence mechanisms can be enhanced by the oral inoculation of young germ free chicks with live E-coli but not with heat inactivated vaccine (Parry and Porter 1981). The studies of Truscott (1981) indicate that there is some degree of protection against gut colonisation after feeding chickens with sonicated lyophilized cells derived from six different serotypes of Salmonella.

The SC of secretory IgA makes it resistant to the effect of proteolytic enzymes and enables it to bind to the apical mucin layer consequently enhancing the biological activity of IgA in vivo (Parry and Porter 1978). Secretory IgA is active against coccidial infection of the chicken through sporozoite agglutination and sporozoite neutralisation (Davis 1981).

In mammals IgA has bactericidal and parasiticidal

activities in conjunction with lysozymes (Newby 1984) but due to the differences that have been found between the secretory immune responses of chickens and mammals (Parry and Porter 1981), it is not possible to assign these functions directly to avian IgA. The role of secretory IgA and its mechanism of action against enteric pathogens, especially Salmonella is not established in the chicken.

(iii) IgM

IgM is produced by B-cells and its production is a characteristic of the primary immune response (Tizard 1982, Glick 1986). After exposure to an antigen the B-cells will divide and differentiate to produce specific IgM against that antigen. This process is controlled by the actions of T-helper and T-suppressor cells (Tizard 1982).

Cells containing IgM in their cytoplasm and on their surface can be seen in the bursa of Fabricius around day 10 (Toivanen et al. 1981) and the bursa starts IgM production from day 14 of incubation (White 1981). Embryonic antibody production does not respond to injection of antigen and IgM is detectable in the serum of young chicks on day 4 after hatching (Rose and Orlans 1981).

In normal chickens, IgM secreting cells are abundant in bone marrow, spleen, lungs and intestinal lamina propria, but there are relatively few of these cells in the peripheral blood, thymus and intestinal epithelium of chickens (Lawrence et al. 1979). Bienenstock et al. (1973) reported the synthesis of IgM in the Harderian gland, bursa, spleen, caecal tonsils and duodenum of chickens and could find it in the crop, glandular stomach (proventriculus), Peyer's patches, bronchi and thymus.

IgM has been isolated from bile (Lebacq-Verheyden et al. 1974, Mockett 1986), seminal plasma (Higgins 1975), egg white

(Rose et al. 1974) and tracheal washing (Chhabra and Goel 1980). According to Rose et al. (1974) and Higgins (1975) IgM is not present in the chicken egg yolk. The concentration of IgM in the serum of the chicken is 0.5-2.55 mg/ml and it is in the form of pentameric and monomeric molecules (Lebacq-Verheyden et al. 1974, Higgins 1975, Schrann and Losch 1986). Tizard (1982) reported that IgM in the birds is either pentameric or tetrameric and that 70% of the blood IgM is in the pentameric form. More information on the physical properties of chicken IgM is given in Table 1. The structure and properties of chicken IgM are similar to those of its counterparts in other species and there is cross reaction between fowl and human IgM. The H chain of fowl IgM is similar to that in the quail and the pheasant and its J chain is similar to the mammalian J chain (Higgins 1975).

In the fowl, the H chain of IgM is antigenically distinct from IgG H chain but light chains are identical (Higgins 1975). IgM is the main agglutinating antibody and is very active in the indirect haemagglutination test (Leslie and Benedict 1968). It is also involved in the immunoprecipitation reaction (Higgins 1975). It is postulated that IgM is a secretory antibody in the alimentary tract of chickens (Lee et al. 1981) as it is in man and other mammals (Newby 1984), but the presence of SC on this immunoglobulin either in the chicken gut or in other secretions has not yet been studied.

(iv) IgG

IgG is the major immunoglobulin class of chicken serum (Higgins 1975, Tizard 1982) with a mean concentration of 5.09 mg/ml (Chhabra and Goel 1980), 5.5 mg/ml (Lebacq-Verheyden 1974) and a range of 0.3 to 12.6 mg/ml at different ages (Higgins 1975). It is the only immunoglobulin present in the egg yolk of the chicken (Rose et al. 1974), pigeon (Goudswaard et al. 1977b) and turkey (Goudswaard et al. 1977a). It is not easy to measure the concentration of IgG in the yolk but it is

higher than the serum concentration and the changes of level of antibody in the blood are reflected in the yolk with a delay of 5 to 6 days (Rose and Orlans 1981). It is estimated that yolk IgG concentration is 10-25 mg/ml and that it has all the properties of serum IgG (Rose et al. 1974, Johnstone and Thorpe 1982). IgG is also present in tracheal washings (Chhabra and Goel 1980), seminal plasma, saliva and tears (Lebacq-Verheyden et al. 1974).

Lebacq-Verheyden et al. (1972b) reported the presence of large numbers of plasma cells containing IgG in the spleen and few in the lamina propria of the intestine of the chicken. This was confirmed by Bienenstock et al. (1973), who also found IgG-producing cells in the caecal tonsils, Harderian gland and bursa of Fabricius but not in the crop, proventriculus, Peyer's patches, bronchus and thymus.

IgG is produced by B-cells under the control of T-helper and T-suppressor cells and appears 4-15 days after introduction to antigen in the serum (Tizard 1982). It is the main antibody in the secondary immune response (Tizard 1982, Glick 1986).

There is antigenic similarity between fowl IgG and quail IgG and to a lesser extent with pheasant IgG. In these three species IgG L chains are more similar than H chains. There is no cross reaction between fowl IgG and human IgG (Higgins 1975) and the fowl IgG molecule is somewhat larger than that of mammalian IgG (Tizard 1982, Benedict and Berestecky 1987). Due to the dissimilarities in physical, chemical and antigenic properties between chicken IgG and mammalian immunoglobulins, Leslie and Clem (1969) proposed the name IgY rather than IgG for it but later it was recognised as the functional and biological counterpart of mammalian IgG (Higgins 1975). Some investigators such as Benedict and Berestecky (1987) describe it as 7S Ig, the term IgG is mostly used in the literature to designate this immunoglobulin in the chicken.

Chicken IgG participates in precipitation and agglutination reactions (Higgins 1975) and like its mammalian counterpart, has a tendency to aggregate and to develop, increased susceptibility to proteolytic digestion (Benedict and Berestecky 1987) but does not bind to the protein A of Staphylococcus aureus (Jensenius et al. 1981, Johnstone and Thorpe 1982) and does not fix mammalian complements (Jensenius et al. 1981).

The presence of two IgG subclasses in the yolk (Rose et al. 1974) and three subclasses in chicken serum (Higgins 1975, Tizard 1982) is claimed but further studies are needed to produce conclusive evidence for this claim. The role of this immunoglobulin in Salmonella infection is not clear and more information is given in Sections 4 and 5 of this Chapter.

(v) IgD and IgE

The presence of these two immunoglobulins has been reported in the chicken (Rose and Orlans 1981, Tizard 1982, Glick 1986, Benedict and Berestecky 1987), but nobody has studied the detailed functions and role of these immunoglobulins in poultry.

In mammals IgD and IgM are present on the surface of some circulating lymphocytes and by their mutual interaction in antigen reception play a role in lymphocyte activation and suppression (Roitt 1988). IgE is present in serum and secretions at very low concentrations and produces a protective response in the intestinal mucosa against parasitic infections (Newby 1984, Roitt 1988). IgE is also held responsible for hypersensitivity after contact with allergens in hay fever and some other allergic diseases (Roitt 1988).

d) Cell mediated immunity

T-cells originating from thymus are the basic components

of cell mediated immunity and they are responsible for the immunological phenomena that are not dependent on antibody synthesis (Glick 1986). Delayed hypersensitivity, graft-versus-host (GvH) reaction, allograft (host-versus-graft response), cytotoxicity and anti-tumour immunity are some examples of cellular immunity (Firth 1977, Tizard 1982, Glick 1986). The thymus is the first organ of the immune system to become lymphocytic during histogenesis of the lymphoid tissue (Manning 1981). The first stem cells enter the thymus around day 7 after incubation in a continuous phase for 36 hours. There is another influx of stem cells to the thymus around hatching time. These cells multiply and differentiate in the thymus and then migrate to the peripheral lymphoid organs (Toivanen et al. 1981). The early functional sign of T-cells within the thymus is demonstrable in the late embryonic period prior to hatching by the GvH reaction (Firth 1977).

The differentiation and responses of T-cells are controlled by soluble factors called lymphokines or cytokines. These factors originate from different cells such as macrophages and T-cells and act upon specific cellular targets. Some of these factors interact with B-cells to regulate antibody synthesis (Schauenstein and Kromer 1987).

The T-cells are responsible for combating intracellular pathogens able to survive and grow in phagocytes (Winter 1979). Macrophages infected with an intracellular parasite release interleukin-1 which is also called lymphocyte activity factor (Schauenstein and Kromer 1987). Interleukin-1 attracts the T-helper cells to bind to the combination of antigen and major histocompatibility complex (MHC) which is formed on the surface of the macrophages and T-helper cells then produce lymphokines (Roitt 1988) composed of gamma interferons and interleukin-2 (Schauenstein and Kromer 1987). The gamma interferons control the activation of the macrophage (Schauenstein and Kromer 1987) by switching on the ability of the macrophage to kill the parasite (Roitt 1988). Interleukin-

2 acts on both B and T-cells. It stimulates the growth and differentiation of B-cells that leads to antibody production and regulates the growth of antigen stimulated T-cells and the differentiation of T-cytotoxic cells (Schauenstein and Kromer 1987).

T-cytotoxic cells will bind to macrophages containing the parasite and produce their cytotoxic effect by killing the infected cells and in this way prevent the multiplication of the pathogen within that cell (Roitt 1988).

The functions of the T-cells in the chicken are similar to those in mammals (Chi and Thorbecke 1987, Schauenstein and Kromer 1987). The T-suppressor cells can either act on B-cells and suppress humoral immunity (Glick 1986) or interfere with other T-cell responses. Chi and Thorbecke (1987) reported that T-suppressor cells are involved in the suppression of immunoglobulin synthesis, proliferative neoplastic cells, anti-tumour immunity, T-cell tolerance and delayed hypersensitivity reactions. The T-cytotoxic cells are responsible for immunity against viral infections, anti-tumour immunity and rejection of allografts (Tizard 1982, Fahey and York 1987).

e) Non lymphoid cells

Macrophages, monocytes, heterophils (equivalents of the mammalian neutrophil), eosinophils, thrombocytes and mast cells are non lymphoid cells that contribute to immunity in avian species. Macrophages are most important as they have a high capacity for phagocytosis and pinocytosis and play a part in both humoral and cellular immunity (Powell 1987). It has been shown that macrophages are involved in the restriction and destruction of viruses, the intracellular killing of bacteria such as Salmonella and E. coli, destruction of malignant cells, killing of extracellular parasites, resistance to fungal infection, delayed hypersensitivity and auto-immunity (Powell 1987).

Popiel and Turnbull (1985) demonstrated the uptake of S. enteritidis and S. thompson by macrophages in the caecal lumen of day old non immune chicks and Mayrhofer (1984) described the function of macrophages in bacterial infection of the gut and suggested that virulent Salmonella are killed after ingestion by the lysosomes of macrophages in the lamina propria. The interaction of macrophages with B and T-cells in the production of antibody and cellular immunity has been described above. Maskell et al. (1987) showed that in S. typhimurium infected mice the initial suppression of growth of the bacteria relied upon the macrophages.

B) Non specific defence mechanisms

Beside the immune responses that described above there are some other factors that affect the outcome of Salmonella infection. The important ones are reviewed here.

a) Normal intestinal microflora

The presence of microflora on the body surfaces including mucous membranes such as those of the alimentary tract, upper respiratory tract, urogenital tract and on the skin are reported. This microflora restricts the growth of exogenous organisms. The importance of these microbial populations in defence against Salmonella infection in the intestine of the chicken is reviewed here.

Young animals and chicks acquire the microflora from their environment after their birth. E. coli, Clostridium perfringens and Streptococci are the first bacteria to colonise the gut followed by Lactobacilli and later on Bacteroides (Woolcock 1979). Stavric et al. (1985) obtained 274 bacterial isolates from the gut contents of adult chicken most belonged to the genera Bacteroides, Lactobacillus, Streptococcus, Escherichia, Eubacterium, Fusobacterium, Clostridium, Bifidobacterium and Propionibacterium or were other Gram positive rods.

The pathogens can be inhibited by the normal microbial flora in different ways. Inhibition could be by competition for attachment sites (Soerjadi et al. 1982, Woolcock 1979), competition for available nutrients, the production of harmful conditions such as unsuitable pH and oxidation reduction potential and production of harmful substances such as toxic metabolites (e.g. H_2O_2) and antibiotics (Woolcock 1979, Newby 1984).

Chickens are more susceptible to bacterial enteric pathogens at the early stage of their life due to a lack of intestinal microflora (Nurmi and Rantala 1973) or after oral antibiotic treatment that destroys the microbial population (Woolcock 1979). Treatment of day old chicks with gut contents from adult chickens (Nurmi and Rantala 1973, Snoeyenbos et al. 1978, Snoeyenbos et al. 1979, Weinack et al. 1980, Soerjadi et al. 1981a and b), fresh faeces of the adult chicken (Snoeyenbos 1978, Silva et al. 1981b), lyophilized extract of breeder flock litter (Rigby and Pettit 1980) followed by subsequent challenge by Salmonella reduced the number of infected birds and the extent of colonisation. This phenomenon is commonly called the "Nurmi effect" or competitive exclusion.

Rantala and Nurmi (1973) found that horse faeces and bovine rumen fluid were not capable of preventing the colonisation of chick caeca by S. infantis. No protection was afforded by Lactobacilli isolated from the crop and caecum of chickens (Soerjadi et al. 1981b). Stavric et al. (1985) showed that competitive exclusion afforded by a mixture of 50 bacteria in pure culture is similar to that obtained with faecal or caecal culture of unknown bacterial composition and reduction of the number of isolates in that mixture led to less protection. Barrow and Tucker (1986) inoculated day old specific pathogen-free (SPF) chicks with a mixture of 3 strains of E. coli and challenged them with S. typhimurium after 24 hours which led to a reduction in Salmonella colonisation. The breed of the chicken and the diets used did not affect this

protection. Inhibition of Salmonella colonisation by members of the same genus has also been reported by Barrow et al. (1987c).

These types of experiments have also been conducted in turkey poults (Snoeyenbos et al. 1978, Reid and Barnum 1983, Weinack et al. 1982) with virtually the same results as described for chicks. The gut contents of chickens and turkeys were cross protective when used in reciprocal trials (Weinack et al. 1982).

The common agreement among all of these investigators is that pretreatment must be carried out before Salmonella exposure for protection to be achieved and optimum protection is obtainable after 32 hours post-treatment (Soerjadi et al. 1981a). There is a risk of introducing enteric pathogens if natural gut flora is used directly in treatment.

b) Diet and feed additives

Although it is claimed that dietary constituents such as proteins may affect the level of enteric bacteria, Hinton et al. (1986) could not find any significant effect of proteins on Salmonella carriage, using diets containing different levels of protein and diets with animal or vegetable proteins from different sources.

In general, therapeutic drugs are not capable of eliminating Salmonella infection in infected flocks while they may reduce mortality and the spread of disease in acute outbreaks. Sulphonamides, antibiotics and nitrofurans have some effect when used as therapeutic measures (Williams 1984). The problem with growth promoters and drugs to which Salmonella are resistant is that they increase Salmonella shedding in quantity and duration in the chicken by destruction of normal flora of the gut (Barrow et al. 1984, Linton and Hinton 1984, Smith et al. 1985). Recently it is reported that formic and propionic

acid can prevent and decrease Salmonella shedding by inclusion in the feed (Hinton and Linton 1988). Williams (1984) reported that Sulphadimethoxine and Ormetoprim in the feed can prevent losses from S. typhimurium infection in poults and reduce the colonisation and shedding of the organism in the chicken.

c) Lactoferrin and lysozyme

Iron is a trace element which is needed by both host and bacteria. Therefore the host tries to limit its use by bacteria by producing iron binding proteins such as lactoferrin and transferrin that bind to iron and render it unavailable to the bacteria. These proteins are found in serum, milk, egg white, seminal fluid, tears, nasal secretion, saliva (Woolcock 1979) and intestinal secretions (Newby 1984). In experimental infection with S. typhimurium, lower LD50s were required and bacterial multiplication is enhanced after supplying the host with exogenous iron (Woolcock 1979). This iron binding may not affect the virulence of intracellular pathogens, but in natural disease its effect on the extent of extracellular growth might be a critical factor (Clarke and Gyles 1986).

Lysozymes are present in the gut and egg white of chickens. Stokes (1984) reported the possible oral adjuvant properties of lysozyme and pointed out that adding egg white lysozyme to babies milk increases levels of the secretory IgA which can be detected in their faeces. While the effect of lysozyme on Gram negative bacteria remains in doubt, this enzyme may play a part in the prevention of Salmonella colonisation in the chicken.

d) Mucus

The mucus produced by Brunner's glands and by goblet cells covers the mucosal surface of the gastrointestinal tract. Several functions have been attributed to the mucus such as lubrication and protection of epithelium from digestive

processes, prevention of the attachment of bacteria to the epithelium, binding to toxins, providing a medium for the functions of enterocytes, lysozyme, IgA and lactoferrin. It may also surround foreign substances and complexes of antigen and secretory antibodies, trapping bacteria by attaching to receptor sites which would otherwise bind to the epithelial cells and diluting the bacterial population by continuous flow into the lumen (Newby 1984).

e) Others

Some other factors contribute to the host defences against bacterial infection such as the epithelial barrier that prevents the penetration of bacteria into the body and the low pH of the proventriculus which is destructive for many bacteria including Salmonella. Intestinal movements also play an important role in reducing the number of bacteria and the extent of their colonisation (Newby 1984).

4 - EGG TRANSMITTED IMMUNITY

IgG is transferred via the placenta to the offspring in some mammals and via the colostrum in others. IgA and IgM are transferred in the colostrum after birth and protect the neonate from pathogens at early stage of their life while they are immuno-incompetent. In birds the conditions are the same except that the immunoglobulins are transferred through their eggs.

The earliest report of egg transmitted immunity was by Klemperer in 1893 who demonstrated the presence of immunity to tetanus toxin in the chicks of vaccinated hens (cited by Brierley and Hemmings 1956, Rose and Orlans 1981). Since then there have been voluminous reports on this subject in general and on antibodies against various avian pathogens. Lancaster (1964) in his review reported the effects of passive immunity in protection and interference with the development of active

immunity against Newcastle virus in chicks. Many investigators have reported egg transmitted immunity to other avian pathogens such as infectious bronchitis virus (Jungherr and Terrell 1948, Winterfield et al. 1980, Mockett et al. 1987), infectious bursal disease virus (Lucio and Hitchner 1980, Lukert and Hitchner 1984), avian encephalomyelitis virus (Luginbuhl et al. 1984), S. pullorum (Beaudette 1923, Buxton 1952), S. gallinarum (Karthigasu 1964) and E. coli (Karthigasu 1964, Heller 1975) in the domestic fowl. Passive immunity to S. typhimurium bacterin and endotoxin in turkey poults was reported by McCapes et al. (1967) and Truscott and Friars (1972) respectively.

The transfer of the immunoglobulins from hens to chicks occurs in two steps. In the first step, IgG is transferred from plasma to maturing oocytes in the ovarian follicle (Kowalczyk et al. 1985) and IgA and IgM are secreted from the oviduct and are acquired by the egg albumin while it is passing through the oviduct (Tizard 1982). In the second step the yolk IgG passes through the yolk sac to the circulation of the growing embryo (Kowalczyk et al 1985) and IgA and IgM are transferred to the amniotic fluid and swallowed by the embryo before hatching (Rose and Orlans 1981, Tizard 1982).

The concentration of yolk IgG is always constant from the smallest oocyst to the mature yolk in the fresh egg and as the yolk mass growth increases from the 8th day before ovulation, the IgG transfer to the yolk also increases (Kowalczyk et al. 1985). These authors also reported that the concentration of this immunoglobulin in the yolk is similar to but lower than that in serum of the hen in contrast with the findings of Rose et al. (1974) who reported higher concentrations of IgG in yolk than in maternal serum. IgG is transferred selectively across the oolemma (Rose and Orlans 1981, Kowalczyk et al. 1985) but it is not clear whether it is receptor mediated or mediated by some other mechanism.

Brierly and Hemmings (1956) showed that the transport of

IgG from the yolk to the chick's circulation is selective. It is detectable in the serum of the embryo from day 7 (Kowalczyk et al. 1985) or day 12 (Kramer and Cho 1970) of incubation and it is agreed by all authors that the rate of transfer increases sharply from day 19 to 21 of incubation.

IgG is absent from the egg white of unincubated eggs, but it is reported in this compartment of incubated eggs from day 4 to day 16 of incubation and also in the amniotic and allantoic fluid and intestine of the embryo (Kramer and Cho 1970).

IgA and IgM of the white are transferred to the embryo intestine via amniotic fluid and there is no indication of their presence in the serum of newly hatched chicks (Rose et al. 1974). As described above these are secretory immunoglobulins that are derived from oviduct secretory cells and there is no evidence for their transfer from maternal serum to the egg.

While it is well documented that there are specific IgG antibodies to some viruses and bacteria in the hen's egg, the presence of specific IgA and IgM antibodies is yet to be confirmed, although it may be taken that specific IgA, IgM and IgG are transferred to the chicks, their effects in the protection of the chick from many pathogens such as S. typhimurium is unknown.

5 - PROTECTION BY VACCINE

In Salmonella infection the virulence of the bacterium must be understood and knowledge of the mechanisms of colonisation, invasion and toxicity is necessary to design appropriate protective vaccines against it (Parry and Porter 1981). Vaccination against Salmonella infections is reviewed below with particular emphasis on salmonellosis of chickens.

A) Types of immunity

The literature is full of claims and counter claims about the possible role of the cell mediated immunity or humoral immunity in protection against salmonellosis. Mitsuhashi et al. (1961) reported that mononuclear phagocytes obtained from the peritoneal cavity of mice, already vaccinated with live S. enteritidis intraperitoneally were able to inhibit the intracellular multiplication of the same organism in vitro. The sera of these mice or cells from mice vaccinated with dead vaccine failed to do so. Collins and Mackaness (1968) showed delayed hypersensitivity reactions in mice vaccinated with live S. enteritidis or S. gallinarum but not with S. pullorum. Killed vaccine of each one of these three organisms failed to produce this type of reaction. The delayed type hypersensitivity was transferable to normal mice by spleen cell transfer. Collins (1968) did not find any evidence for a major role of humoral factors in cross protection against intravenous injected S. enteritidis in mice already vaccinated with 6 strains of Salmonella, 2 strains of E. coli and some other bacteria.

Using temperature sensitive mutant strains of S. enteritidis Cooper and Fahey (1970) orally immunised rats and did not find protective humoral immunity in the intestine. Cameron and Fuls (1974) postulated the possible involvement of both types of immunity in the immunogenicity of live and inactivated S. typhimurium vaccine in mice. Cameron (1976) suggested a prominent role for cellular protection against infection in the chicken, but he did not rule out the possible participation of serum antibodies in protection.

The idea of the importance of cell mediated immunity against Salmonella infection in the chicken was also proposed by Fahey and Cooper (1970b), Padmanaban et al. (1981), Lee et al. (1981), Silva et al. (1981a) and Lee et al. (1983). Most of these workers believed that clearance of Salmonella from

chicken tissues is mediated by cellular immunity and all agree on the development of humoral immunity after vaccination or natural infection, but they were doubtful about the protective effect of antibodies.

In contrast to the work described above, some other workers have shown the involvement of humoral immunity in the protection against Salmonella. Morris et al. (1976) used immunosuppresive agents and were able to study the role of humoral and cell mediated immunity individually in mice. The sera of mice vaccinated with a gal-E mutant strain of S. typhimurium were transferred to mice that had suppressed B and T-cell systems. This serum transfer resulted in protection against the challenge organism. Mice with suppressed B-cell systems and intact T-cell systems could not survive the challenge. They concluded that humoral immunity is a key factor in the protection of mice against this strain of S. typhimurium.

Observations of Schauenstein and Kromer (1987) showed that the LPS of E coli and Salmonella only stimulate B-cell proliferation. According to Woolcock (1979) high concentrations of antibody are needed for the destruction of virulent intracellular S. typhimurium and antibody and complement are needed to assist both phagocytosis and other antibacterial activities against this organism. Further evidence comes from studies of salmonellosis in poultry. McCape et al. (1967), Truscott and Friars (1972) and Thain et al. (1984) demonstrated protective effects of passive immunity against Salmonella infection in poults derived from vaccinated hens, thus demonstrating the importance of antibodies in this situation.

B) Types of vaccine

A number of different types of vaccines have been given by different routes to assess their protective value in animals and birds. Botes (1965) reviewed the different aspects of

immunisation in relation to vaccine development in poultry and calves. According to this author the dead Salmonella vaccines are not efficient and that formalin killed vaccine for S. gallinarum is potent when it is fresh, while storage for 6 weeks reduces the potency of this vaccine. He pointed out that a live attenuated vaccine of S. typhimurium keeps its protecting value until it is virulent and loss of virulence causes a considerable loss in immunising potency.

Storage of formalin killed S. typhimurium at 50°C for one month did not affect the immunising potency of the vaccine in mice (Cameron and Fuls 1974). Germanier (1970) used different rough mutants of S. typhimurium in the vaccination of mice by the SC, IV and oral routes and showed that there was no correlation between immunising potency and the virulence of these organisms. Immunising potency depended on the persistence of viable bacteria in the mouse.

Temperature sensitive mutants of S. enteritidis that are not able to proliferate inside the body of the mouse (37°C) when given orally are capable of inducing protective immunity against subsequent oral infection (Fahey and Cooper 1970a). Knivett and Stevens (1971) reported that a live attenuated vaccine of S. dublin was able to reduce the number of challenge S. typhimurium and that both oral and SC routes of vaccination were equally effective. Protection afforded was independent of the size of the vaccine dose.

The degree of cross protection depends on the serotypes of Salmonella concerned and the species of animal used for vaccination (Cameron 1976). This author also showed that live or inactivated vaccines of S. typhimurium and S. dublin are equally effective in mice and calves and suggested that inactivated vaccines were most advantageous. The use of a live mutant strain of S. typhimurium in the vaccination of chickens to provide some degree of protection has been reported by Pritchard et al. (1978) and Suphabphant et al. (1983).

Angerman and Eisenstein (1980) used acetone killed cells, LPS, ribosomes and live cells of S. typhimurium in the vaccination of different groups of mice and challenged them with the same organism at different time intervals. They found that the ribosomal vaccine could induce longer lasting immunity than the others, while LPS vaccine was the least effective one of all. Alkali treatment of S. typhimurium LPS reduces its immunological reactivity (Ciznar and Shands 1970). Proteins of Salmonella are major immunogens and have a protective effect in the vaccination of mice that is cross protective against other Enterobacteriaceae (Barber and Eylan 1976).

Truscott and Sajnani (1972) used endotoxin and endotoxoid of E. coli and S. typhimurium in the vaccination of chickens and found that there was cross protection among the species within a genera. Best results were obtained by IV injection of the vaccine when compared with the IP and SC routes. Endotoxoid alone was unable to produce protective immunity but it had a synergistic effect when used in conjunction with endotoxin. Chicks less than two weeks old produced poor antibody responses to endotoxin injection and older birds produced higher antibody levels than younger birds with the same dose of antigen. Sonicated freeze dried antigens of different Salmonella serotypes were able to provide a local protection in the gut when administered by the oral rout (Truscott 1981).

6 - CONCLUSIONS AND OUTLINE OF THE EXPERIMENTAL APPROACH

S. typhimurium is a pathogen of zoonotic importance and infects eggs, meat and other poultry products which are potential sources of human food poisoning. It is also the causative agent of poultry salmonellosis and produces huge economic losses around the world in all phases of the poultry industry. Attempts to control poultry salmonellosis by treatment, hygiene, competitive exclusion, vaccination and genetics have not been completely successful. The mechanism of immunity to Salmonella infection is not well established and

the role of different immunoglobulins in possible protection against infection is virtually unknown. Different vaccines have shown some protective effects in direct challenge with Salmonella. Chicks are immuno-incompetent at the early stage of their life in the same way as young mammals. Their immune system is not well developed and they are helpless against Salmonella pathogens that can infect them in the hatcheries or later on after hatching. It is clear that egg transmitted immunity is capable of protecting young chicks against some viral infections at this stage of life. On the bacterial side, studies of different bacteria including Salmonella in turkeys, raise hopes of the possible value of passive immunity in the protection of chicks.

As a result of the information on this world wide problem reviewed above, it was decided to study various aspects of S. typhimurium pt. 49 infection in the chicken. First the capability of the organism to colonise the chicks' intestine and its role in provoking antibody production in naturally infected birds was determined. The possibility of the transfer of specific immunoglobulins to different compartments of the egg and the role of passive antibody in the protection of day old chicks were investigated. These studies were followed by an attempt to hyperimmunise hens to provide the maximum levels of egg transmitted antibody. Accordingly SPF hens were vaccinated with formalin killed vaccine plus oil adjuvant supplemented with oral sonicated freeze dried antigen. The immune response of these birds and the transfer of antibodies to the eggs were studied. The degree of protection against challenge with the same organism was studied. Finally antibodies isolated from egg white and yolk were transferred to embryonated eggs obtained from SPF hens in order to evaluate the ability of each antibody to protect newly hatched chicks against challenge with the organism.

CHAPTER 2

MATERIALS AND METHODS

1 - BACTERIOLOGICAL STUDIES

A) Media

The following media were used throughout this study.
Sheep Blood Agar: Oxoid blood agar base No.2 with added 7% formalised sheep blood (C.C. Laboratories).
MacConkey Agar (Oxoid CM7).
Modified Salmonella Shigella (SS) Agar (Oxoid CM533).
Hynes modification of Desoxycholate Citrate Agar (DCA) (Oxoid CM227).
Brilliant Green (BG) Agar (Oxoid).
Tetrathionate Broth Base (Oxoid) with 0.2 ml of an iodine in potassium iodide solution added to every 10 ml (Oxoid manual 1982).
Triple Sugar Iron (TSI) Agar (Oxoid).
Nutrient Broth (Oxoid No.2).
Urea Broth: Urea broth base (Oxoid) with 5% added sterile 40% urea solution (Oxoid SR20).
Peptone Water (Oxoid L32).

All of the above media were prepared according to the supplier's instructions. The agar media were dispensed in sterile disposable plates (Sterilin Ltd., Feltham, England) and the broth media were aliquoted into universals or other appropriate containers. Others were made up as follows:

Tryptone Soya Agar (TSA) (Oxoid): This medium was made up as described by the Oxoid manual, but dispensed in 800 ml Roux flasks (Nunclon^{R}). Each flask contained 135 ml of the prepared medium (Truscott, 1981).

Sloppy Agar: 0.6% of agar bacteriological No.1 (Oxoid L11) was

added to a 2.5% solution of nutrient broth (Oxoid). It was dispensed in 10 ml aliquots into universals and sterilised. Tubes intended as Craigie tubes (0.6 cm wide and 2cm long) were sterilised separately and one was inserted into the medium of each universal under sterile conditions. About 0.5 cm of the tube was left out of the medium. The medium was inoculated through this tube.

B) The organism and storage conditions

Salmonella typhimurium phage type (pt.) 49 was provided by the Scottish Salmonella Reference Laboratory, Stobhill Hospital, Glasgow. It was isolated from a colonised broiler flock. The organism was supplied on a nutrient agar slope and received within a week of isolation. It was subcultured onto sheep blood agar and MacConkey agar to confirm the purity of the culture. One colony from the sheep blood agar was subcultured further onto 5 sheep blood agar plates and incubated at 37^{o}C overnight. The culture was tested by slide agglutination test for O, H and B group Salmonella antigens, using Salmonella agglutinating sera (Wellcome Diagnostics, Dartford) as described in Identification of Salmonella below. The remainder of the bacterial growth was harvested in 5 ml of a freeze drying solution, prepared according to a method used in the department and given below.

5.77g of glucose (Farmachem) and 1.5g peptone water powder (Oxoid) were dissolved in 100 ml deionised water and sterilised by positive pressure filtration through a cellulose acetate filter of APD 0.22 mm (Millipore). 15 ml of sterile horse serum (Gibco) was added per 100 ml. The bacterial suspension was dispensed in 0.25 ml amounts into freeze drying ampoules and freeze dried using an Edwards freeze drier (Edwards, Colchester). Ampoules were sealed under vacuum and stored at 4^{o}C until required.

C) Purity test

It was necessary to confirm the purity of cultures at intervals. The confirmation process was carried out by inoculation onto sheep blood agar, MacConkey agar and brilliant green agar plates. Inoculated plates were incubated at 37°C for 48 hours. The resulting growth was examined and the culture was not used if contamination was suspected.

D) Sterility test

Vaccines were tested for sterility before use. In this case sheep blood agar, MacConkey agar, brilliant green agar and tetrathionate broth were used. All were incubated at 37°C for 48 hours. After 24 hours and 48 hours plates were examined and tetrathionate was subcultured onto DCA, SS and BG agar plates. These plates also were incubated for 48 hours and examined after 24 and 48 hours. Vaccines were discarded if there was any growth on any one of these plates.

E) Production of flagellated organisms and confirmation of the presence of flagella and fimbriae

Flagellated organisms were produced for vaccine and immunological studies by the following method. The contents of one freeze dried ampoule were dissolved in one drop of sterile peptone water and were tested for purity. One colony from the MacConkey agar plate was subcultured into sloppy agar (inside the Craigie tube)(Bettelheim and Maskill, 1985) and incubated at 37°C for 48 hours. Uninoculated sloppy agar was also used at the same time as a control. Purity and the presence of flagella were confirmed and the flagellated organisms were kept in a universal of sloppy agar at 4°C until used.

For confirmation of the presence of flagella and fimbriae the organisms from sloppy agar were used directly or after they were subcultured into nutrient broth and incubated at 37° C,

overnight in an orbital incubator (Gallenkamp, England) with the speed of 140 rev/min. A drop of this culture was placed on a slide and covered by a cover slide and examined for motility by light microscopy (Leitz, West Germany). Negatively stained preparations were made from these cultures by placing a drop of the culture on a parlodion coated copper grid, and stained with 2% phosphotungstic acid. They were examined by electron microscopy (Zeiss 109, Oberkochen, West Germany) for the presence of flagella (Fig. 4) and fimbriae(Fig. 5). The electron microscopy (EM) was carried out with the help of the staff of the Electron Microscopy Laboratory.

F) The isolation and identification of Salmonella

a) Samples

The samples examined were faeces, litter, feed, water, eggs, cloacal swabs and internal organs of chickens such as heart, liver, alimentary tract, spleen, yolk sac, gall bladder and oviduct. All were examined immediately after collection.

b) Methods of sampling and culture

(i) Feed and litter

10 g of feed or litter was cultured in 100 ml of tetrathionate broth and incubated at 37^{o}C for 48 hours. The tetrathionate broth was subcultured onto SS, DCA and BG agar plates both after 24 and 48 hours of incubation. These plates were incubated at 37^{o}C for 24 hours and examined for the presence of Salmonella.

Feed samples were taken from the top, middle and bottom of feed bags aseptically immediately after the bags were opened. Litter samples were taken from at least 5 different places in a pen and mixed.

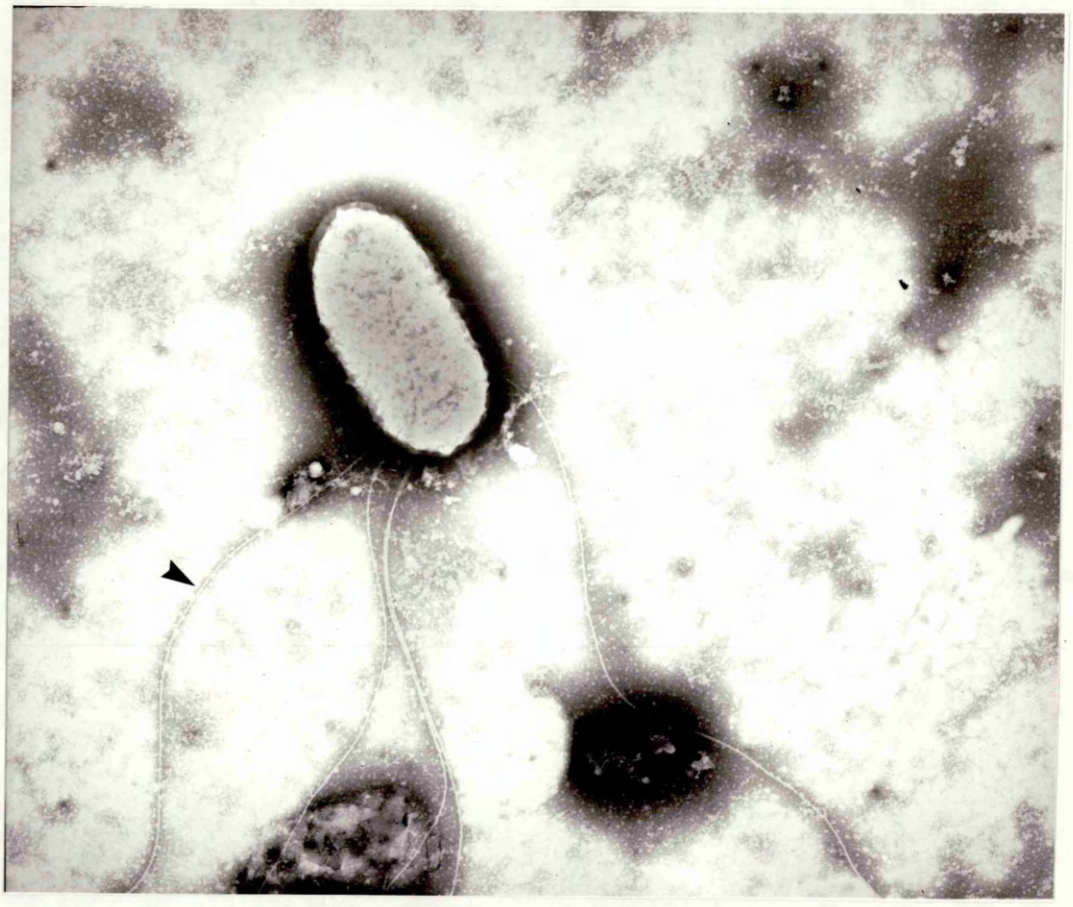


FIG. 4: Electron micrograph of single flagellated cell of S. typhimurium pt. 49 from a 16 hour nutrient broth culture. 4 flagella are present (arrow).
x 24000

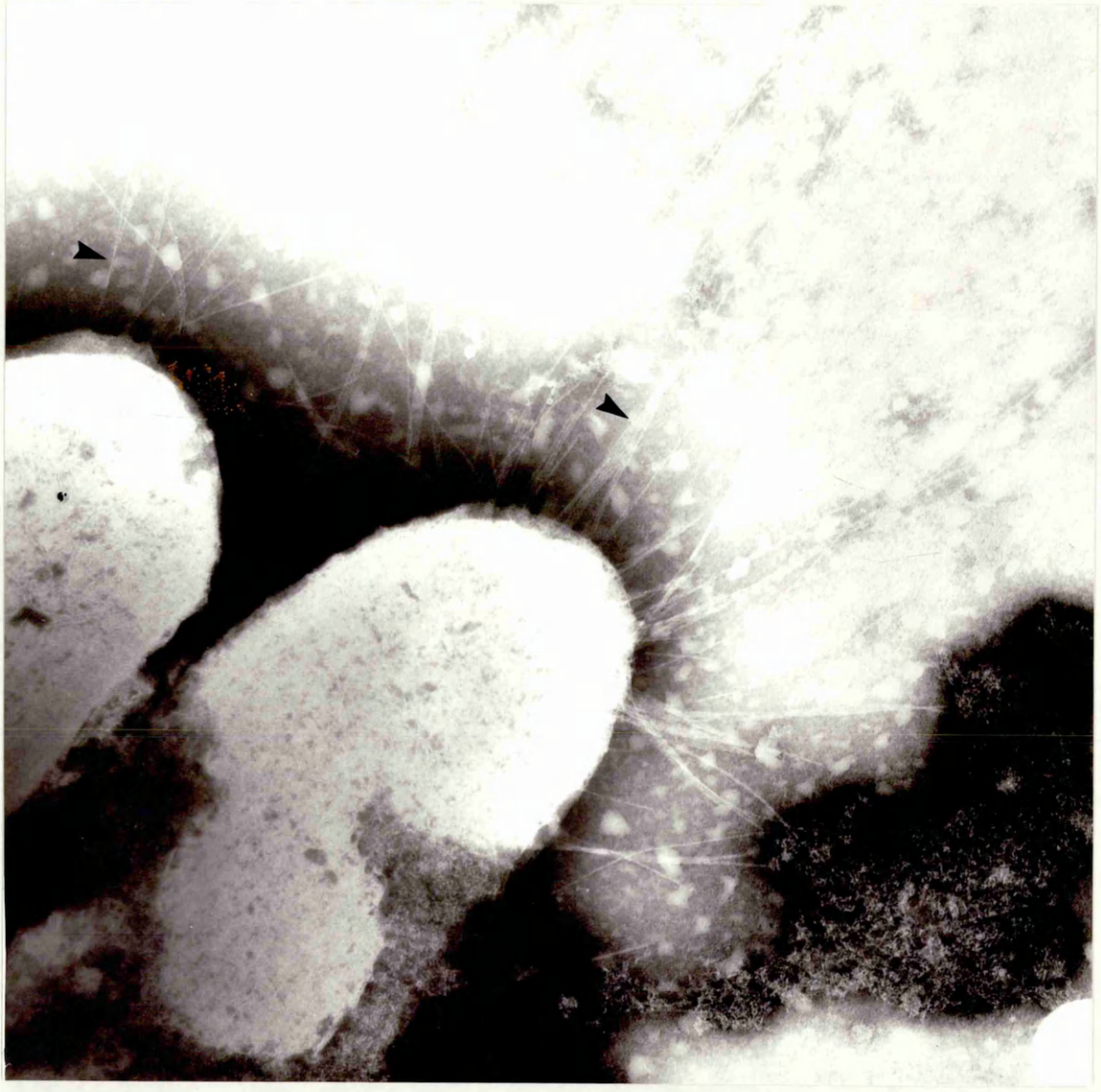


FIG. 5: Electron micrograph of a single fimbriated cell of S. typhimurium pt. 49 from a 16 hour nutrient broth culture. Note the fimbriae (arrows).
x 80000

(ii) Water

The water used by all animals in this study was Glasgow city tap water. Samples of 5 ml were added to 50 ml of tetrathionate broth once at the time of each experiment and monitored for the presence of Salmonella as described above.

(iii) Fresh faeces

10-20 fresh droppings were transferred into a sterile plastic universals (Sterilin Ltd.), using a sterile scalpel blade and mixed thoroughly using a sterile swab. One gram of this mixture was added to 10 ml of tetrathionate broth and cultured for Salmonella by the method described above.

Direct culture on sheep blood agar, MacConkey agar and BG agar was used to test for the presence of other bacterial pathogens. These plates were incubated at 37°C for 24 hours and examined using colonial morphology and Gram stained smears of suspicious colonies to identify pathogens.

(iv) Eggs

Eggs used for bacteriological monitoring were mostly unhatched incubated eggs. All unhatched eggs were first transferred to the cold room (4°C) and were left for at least 4 hours. Then surface of the egg shell was disinfected using absolute alcohol or tincture of iodine and the shell was cut off aseptically at the blunt end using sterile scissors. If there was no embryo, the contents were mixed and cultured in tetrathionate broth and on sheep blood, MacConkey and BG agar plates. When an embryo was present, the yolk sac was snipped with sterile scissors and a large sample of yolk was taken using a sterile loop. This was incubated in the media described above (Williams et al. 1980). After incubation these media were examined for Salmonellae and other bacteria as described in the section, "Fresh faeces".

Unincubated eggs were cultured as described for unembryonated eggs above.

(v) Cloacal swabs

Pernasal transwabs with Amies clear medium (Medical Wire & Equipment Co., Wiltshire, U.K.) were used for young chicks and transwabs with charcoal containing medium (Medical Wire & Equipment Co.) for pullets and adult chickens. Swabs were inserted by gentle rotation into the cloaca of a chicken and then withdrawn, placed into the tubes holding the media and transferred to the lab. Each swab was first cultured onto sheep blood, MacConkey and BG agar plates and then cut into a universal containing 10 ml of tetrathionate broth. Incubation and subculturing was carried out as above.

(vi) Internal organs

Liver, heart, spleen, oviduct, gall bladder, the contents of intestine, caeca, crop and yolk sac were all used for Salmonella monitoring studies.

Immediately after euthanasia the abdominal cavity was opened aseptically and these organs revealed. The surface of each organ was seared with a hot spatula, an incision was made in the seared area and samples were taken under sterile conditions. Samples were aspirated from the heart and gall bladder using a sterile pasteur pipette or sterile syringe and needle.

All samples were inoculated onto sheep blood, MacConkey and BG agar plates and into tetrathionate broth and incubated and monitored as outlined above.

c) Identification of Salmonella species

(i) Colonial morphology

Non lactose fermenting colonies on each medium with morphological characteristics similar to those described in chapter 1 were considered to be Salmonella and were tested further.

(ii) Cellular morphology

In early studies Gram staining of heat fixed smears from suspicious colonies were examined by light microscopy for 1-3 um long, gram-negative bacilli.

(iii) Confirmation by biochemical tests

Colonies suspected of being Salmonella were further tested in urea broth medium for urease production. After inoculation the medium was incubated at 37°C for a period of 5-18 hours. Organisms that produced a red or pink colour in the medium were regarded as not belonging to Salmonella group and were discarded. Organisms which caused no colour change in the medium, were inoculated onto TSI agar slopes and incubated at 37°C for 24 hours. Acid reaction (yellow colour), gas and H_2S in the butt and alkaline reaction (red colour) in the slant were assumed to indicate the presence of Salmonella species and these isolates were examined serologically for further identification.

(iv) Serological tests

The slide agglutination test was carried out by emulsifying the bacterial growth from the surface of TSI agar slants in a drop of physiological saline on a slide to form a dense milky suspension. Then a drop of Salmonella polyvalent O antiserum (Wellcome Diagnostics, Dartford) was added, mixed and

rocked at room temperature. Agglutination within one minute of the addition of antisera was regarded as positive.

The procedure was repeated using anti-flagellar (H) antiserum and specific Salmonella somatic group (A-E) antisera (Wellcome Diagnostics, Dartford), to confirm the antigenicity and determine the serogroup of the Salmonella respectively.

G) Salmonella counting from faeces and gut contents

Caecal contents were used for Salmonella counting in most cases, but the contents of other parts of the gut and fresh faeces were also used. Fresh faeces was collected as described above. Gut contents were collected aseptically and placed in a universal. All samples were kept at $4^{o}C$ just after collection until used.

A suspension of 10% w/v of each sample was made in sterile phosphate buffered saline and serially diluted by ten fold dilution up to 10^{-10} dilution. For each dilution one BG agar plate was used to culture six individual 10 ul drops of that dilution. Plates were marked and incubated at $37^{o}C$ for 24 hours. Distinct colonies of Salmonella were counted at the lower possible dilution and the original number of organisms in one gram of sample was calculated.

H) Preparation of inocula for day old chicks

Salmonellae from sloppy agar were subcultured into 100 ml bottles of nutrient broth and incubated at $37^{o}C$ for 16 hours in an orbital incubator (140 rev/min.). The number of organisms in each ml of this solution was found by making serial ten fold dilutions in sterile phosphate buffered saline and culturing 10 ul drops on BG agar plates. This procedure was repeated several times to get an idea of the total number of colony forming units (CFU) of the Salmonella in each ml of this type of culture.

A fresh culture was prepared for each experiment and diluted to the required dilution. After the chicks were dosed, the exact number of CFU in the fresh inoculum was found by culturing on BG agar plates.

2 - IMMUNOLOGICAL METHODS

The immunological tests and methods used in this study included enzyme linked immunosorbent assay (ELISA), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), immunoelectrophoresis, double immunodiffusion and gel filtration.

A) Enzyme linked immunosorbent assay

A double antibody indirect heterogeneous enzyme linked immunosorbent assay (ELISA) was adapted for chickens to determine the distribution and levels of IgA, IgM and IgG antibodies to the surface protein and lipopolysaccharide antigens of S. typhimurium pt. 49, in serum, bile, oviductal secretions, egg white and egg yolk. The materials used , their preparation and construction methods are described below.

a) Materials used and their preparation

(i) Test samples

Samples of serum, egg yolk, egg white, bile and oviductal fluid from chickens were prepared as follows:

Serum: 1-2 ml of blood was collected either from the wing vein or heart by the methods described in Section 4 of this Chapter. Blood was left to clot at room temperature for 2 hours, ringed and kept at 4°C overnight. Serum was separated and centrifuged at 2000 g for 20 minutes at 4°C, aliquoted and kept at -20°C for further use (Johnstone and Thorpe 1982).

Egg white and egg yolk: Both were separated from each other after breaking the shell and were kept in two separate containers. Egg yolk was diluted by adding an equal volume of PBS and then both yolk and white were homogenised separately using a laboratory mixer and homogeniser (Sorvall, Model OMNI - Mixer 17106, Newtown, Conn.), at low speed for 20 -30 seconds. Each was then diluted to working dilution with diluting buffer (p. 68) and used in the ELISA.

Bile: Immediately after killing the chicken, the abdominal cavity was opened, bile was aspirated from the gall bladder and divided into two portions. One was kept in 4^{o}C and used in the ELISA within 24 hours, and the other was kept at -20^{o}C to determine the effect of freezing on the preservation of bile immunoglobulins.

Oviductal secretions: Immediately after the euthanasia of each hen, the oviduct was removed carefully and completely. 10 ml of sterile PBS was injected via one end and the organ was turned up and down several times, while holding both ends firmly. The contents were then poured into a universal and centrifuged at 2500 g for 30 minutes at 4^{o}C to remove large particles. The supernatant was kept at 4^{o}C and the ELISA was carried out within 48 hours to determine the level of IgA and IgM antibodies to surface protein and lipopolysaccharide antigens of Salmonella. The remainder of the fluid was frozen and kept at -20^{o}C until required.

(ii) Antigens

Surface protein antigen: This antigen was produced according to the method of Parton (1975). S. typhimurium pt. 49 was subcultured from sloppy agar into 1 litre of nutrient broth in a conical flask and incubated at 37^{o}C for 16 hours. The flask was shaken at 140 rev/min. The resultant growth was centrifuged using a Beckman Model J2-21 Centrifuge (Beckman Instruments Inc., Palo Alto, California) at 4000g for 30 minutes at 4^{o}C.

The pellet was kept and washed once with sterile normal saline and resedimented using the same conditions. The cells were resuspended in 50 ml of cold saline, blended in a Laboratory Mixer & Homogeniser at full speed for 2 minutes. The container was kept in ice during the process. The sheared cells were centrifuged at 10,000 g for 30 minutes at 4°C. The pellet was discarded and the supernatant centrifuged at 100,000g using a Sorvall OTD 50 Ultracentrifuge (Sorvall Products, Wilmington, Delaware, USA) for 1 hour at 4°C. The pellet of protein antigen was resuspended in 5 ml of normal saline and 0.5% formalin was added and kept at 4°C for 24 hours. The concentration of total protein was determined by Lowry's method using a Sigma protein assay kit (Sigma Diagnostics, Dorset, England) and was then adjusted to 0.8 mg/ml and dispensed into 50 ul fractions in small vials which kept at -20°C until required.

Lipopolysaccharide antigen: A phenol water extract (PWE) was prepared according to the methods of Luderitz et al. (1966) and Donachie and Jones, (1982).

S. typhimurium pt. 49 from sloppy agar was grown in one litre of nutrient broth for 16 hours at 37°C, with shaking at 140 rev/min. Cells were harvested by centrifugation at 4000 g for 30 minutes and washed twice, each time with 100 ml of sterile distilled water and resedimented. Cells were harvested from one litre of broth culture were then suspended in 50 ml of distilled water and warmed to 68°C in a water bath(Grant Instruments Ltd., Barrington, Cambridge, England). 80% phenol (BDH Chemicals Ltd.,) was added to give a final concentration of 45% phenol. The mixture was kept at 68°C for further 10 minutes and was shaken vigorously. Then the mixture was cooled quickly to 4°C in an ice bath and centrifuged at 9000g for 30 minutes at 4°C. The aqueous layer was removed and dialyzed against running tap water for 24 hours in a semipermeable dialysis bag (Medical International Ltd., London). Dialysate was centrifuged at 100,000 g for two hours at 4°C and the pellet was resuspended in 5 ml of sterile distilled water.

The concentration of lipopolysaccharides was determined by lyophilising 1 ml of this solution and determining the weight of dried material present. The concentration of the remainder of the solution was adjusted to 0.2 mg/ml and was dispensed in 50 ul aliquots in small glass vials and kept at -20^{o}C until required.

(iii) Buffers

Coating buffer: Carbonate bicarbonate buffer pH 9.6 was prepared by dissolving 0.75g sodium carbonate, and 1.46g of $NaHCo_3$ in 500 ml of deionised water and kept at 4^{o}C until used.

Washing buffer: This buffer was composed of 0.106% w/v Na_2HPO_4 anhydrous (BDH), 0.039% w/v $NaH_2PO_4,2H_2O$ (Farmachem), 0.85% w/v sodium chloride and 0.05% v/v Poly Oxyethylene Sorbitan, Monolaurate (Tween 20, Sigma Chemical Company Ltd.) in deionised water at pH 7.2. It was kept at 4^{o}C until used.

Diluting buffer: 0.25% bovine serum albumin (Sigma) and 5% skimmed milk powder (Marvel, Premier Brands U.K. Ltd.) were added to one litre of washing buffer which was freshly prepared prior to use.

Substrate buffer: Phosphate-citrate buffer pH 5.0 (called substrate buffer from this point) was made using 25.7 ml of 0.2 M dibasic sodium phoshate (BDH), 24.3ml of 0.1 M citric acid (BDH) and 50 ml deionized water (Sigma 1987).

(iv) Solid phase (Micro-ELISA Plates)

Two types of 96 well flat bottom immulon (polystyrene) plates were used during this study. They were called M129 A and M129 B plates, both supplied by Dynatech Laboratories Ltd., Sussex, England. M129 A is designed for protein binding in enzyme immuno assay (EIA) and radio immuno assay (RIA). It was used for surface protein antigen binding. M129 B is for general

antigen binding in EIA and RIA and was used for lipopolysaccharide antigen binding in this study. Both plates were of the rigid type.

(v) Antisera

Fc specific anti-chicken IgA, IgM, and IgG were purchased from Nordic Immunological Laboratories Ltd., Maidenhead, Berks, England. They were originally raised in the goat and supplied in lyophilised form, reconstituted to 1 ml with sterile distilled water and dispensed in 50 ul aliquots in small glass vials and kept at -20^{o}C until used.

(vi) Conjugate

This was anti-goat heavy and light chain IgG raised in rabbit, conjugated with horse radish peroxidase (code RAG/IgG(H+L)/PO) and purchased from Nordic Immunological Laboratories. It was reconstituted and dispensed as described above.

(vii) Substrate and chromogen dye

The substrate used during the experiment was hydrogen peroxide (BDH) at a concentration of 0.012% w/v in substrate buffer. Ortho-phenylene diamine (OPD) (4 mg tablets, Sigma Chemical Company Ltd., Dorset, England.) was used as a chromogen dye. It was dissolved in substrate buffer at a concentration of 0.04% w/v or one tablet per 10 ml of buffer . This solution was prepared immediately before use by first adding the tablet and then the hydrogen peroxide to the substrate buffer.

(viii) Micro-ELISA reader

A Micro-ELISA Autoreader MR 580 (Dynatech Laboratories Inc., Virginia, USA) was used to read the optical density of

colour development on both types of ELISA plates. A dual wave length mode was used to assess the absorbance value, using a 490 nm test filter and a 630 nm reference filter. The test wave length (492 nm) was recommended by the supplier of the OPD tablets (Sigma) and the reference wave length was determined according to the Micro-ELISA Autoreader instruction manual.

b) Construction and standardisation of ELISA

The idea for this ELISA was derived from the work of Burrells et al. (1979), Burrells and Dawson (1982), Donachie and Jones (1982), Bettelheim and Maskill (1985), Appassakij et al. (1987), Barclay and Scott (1987) and Mockett et al. (1987).

The ELISA consisted of six steps and four washes with washing buffer. The optimum working dilution of antigens, antisera and conjugated anti-species antisera was determined by chequerboard titration. Serum of a naturally infected hen that was positive in the slide agglutination test was used as a positive standard, and serum of a specific pathogen free hen was employed as the negative standard.

First the positive and negative sera were applied at a fixed dilution on coated plates with each antigen separately and then antiserum was diluted across the horizontal rows and the conjugate was diluted across the vertical columns. The experiment was continued by adding substrate and incubating for 3o minutes at 25°C. The reaction was stopped with 0.5 M H_2SO_4. Colour development on the plates was read by the Micro-ELISA reader using the dual wave length described above. The well with the maximum readable absorbance, the lowest concentration of conjugate and the highest concentration of antiserum was chosen by consulting the printout. The dilution of conjugate at this point was regarded as a relative dilution and was used until the optimum working dilution found. Then the chequerboard titration was repeated for each antigen and antiserum. This time maximum readable absorbance was chosen at

a well with the lowest concentration of antiserum and highest concentration of antigen. At this stage the relative dilutions of conjugate and antisera were found. Then the ELISA was repeated under different temperature conditions and different lengths of time for each step as recommended by Burrells and Dawson, (1982). Finally, when the optimum conditions had been found, the test was repeated by titrating each one of the reagents independently to find the optimum working dilution. On the basis of the data that was obtained for every reagent, a curve was plotted and the maximum readable absorbance or the concentration at the end of the plateau of the curve was selected as the optimum working dilution. Some of the standard curves are shown in Appendix 1. After standardising all of the antigens, antisera and conjugates in this way the ELISA was employed to test the samples as follows:

Step 1: Optimum concentration (working dilution) of lipopolysaccharide was found to be 1 ug/ml and that of surface protein antigen 4 ug/ml of coating buffer. Each of the 50 ul frozen antigen vials described above was thawed and added to 10 ml of the coating buffer to make these dilutions separately. 50 ul of the prepared dilution was applied to each well and incubated at $4^{o}C$ overnight. During incubation in this and all other steps, the plates were covered with cling film to prevent loss of liquid and drying of the wells.

After steps 1-4 the plates were washed three times at each step with washing buffer and beaten against a bundle of wiping tissue to shake off the small water droplets.

Step 2: Samples were applied in duplicate wells and diluted by two fold dilutions across the columns or rows. In most cases, an initial dilution of each sample was made in diluting buffer and placed in the wells and further diluted. This improved reading of the end points. The initial dilution differed for different samples and is shown in Table 2. These dilutions were used unless otherwise indicated.

Table 2

Initial dilutions of the samples examined in the ELISA during the study.

Sample	Chicken groups used in the study				
	Naturally infected	Orally vaccinated	Paren.-Oral. vaccinated*	SPF	Young chicks
Serum IgG	1:300	1:300	1:1200	1:300	1:300
Serum IgA+	1:100	1:100	1:200	1:100	1:100
Yolk	1:320	1:320	1:640	1:320	---
Egg white	1:20	1:20	1:40	1:20	---
Oviduct secretions	As prepared	As prepared	As prepared	As prepared	---
Bile	1:80	1:80	1:80	1:40	---

* = Parenterally-orally vaccinated.

+ = The initial dilution for IgM measurement was the same as those of IgA.

At this stage the plates were incubated in a hot room at 37°C for 30 minutes while they were shaken on a shaker (Luckham Ltd.) at medium speed.

Step 3: Plates were washed and and dried as described above. 50 ul of goat anti-chicken antiserum was added to each well and the plates were covered and incubated in the conditions as described in step 2. The antisera used were anti-chicken IgA or IgM or IgG (Fc specific) and the optimum working dilution for each one was 1:400, 1:800 and 1:5000 respectively.

Step 4: Plates were washed and dried as above. Rabbit anti goat IgG (H+L chain) conjugated with horse radish peroxidase enzyme was diluted to optimum working dilution (1:10,000) in diluting buffer and 50 ul added to each well. The plates were covered and incubated as above.

Step 5: The plates were washed and dried as before. 50 ul of freshly prepared substrate was added to each well. The plates were incubated at 25°C for 30 minutes in the dark.

Step 6: 50 ul of 0.5M H_2SO_4 was added to each well to stop the reaction and the absorbance of colour development was read by the Micro-ELISA Autoreader at 490 nm (A_{490}) as described above.

c) Validation of the ELISA

The ELISA was then tested for its specificity and sensitivity. Firstly the antigens were examined for purity using SDS PAGE.

(i) Sodium dodecylsulphate polyacrylamide gel electrophoresis

Methods of gel preparation and electrophoresis: Surface protein and lipopolysaccharide antigens prepared for the ELISA were electrophoresed on a sodium dodecylsulphate (SDS) polyacrylamide gel according to a method used in the department

(A.N. Rycroft, personal communication) modified from that described by Johnstone and Thorpe (1982).

A mixture of proteins was used as reference standards (SDS molecular weight markers, Sigma Chemical Company) and consisted of carbonic anhydrase, egg albumin, bovine albumin, phosphorylase b, beta galactosidase, myosin with added lysozyme and trypsin inhibitor.

40 ml of running (separation) gel was made using 12.5% (16.7 ml of 30% stock solution) of acrylamide and 0.1% N,N'-methylene bisacrylamide (2.0 ml of 2% stock solution), 14.9 ml of 1M Tris HCl (pH 8.7), 0.2 ml of 20% SDS (final concentration of 0.1%), 30 ul of N,N,N,N'- tetramethylethylene diamine (TEMED), 13.5 mg (135 ul of 10% solution) of ammonium persulphate and 6.2 ml of distilled water. All were mixed together except the TEMED and ammonium persulphate, which were added after degassing of the rest of the solution. 10 ml of stacking gel was made from 5.1% acrylamide (1.7ml of 30% solution) and 0.14% of N,N - methylene bisacrylamide (0.7ml of 2% solution) 1.25ml of 1 M Tris HCl (pH 9.6), 50 ul of 20% SDS, 152 ul TEMED, 50 ul ammonium persulphate and 6.35 ml of distilled water. Stacking gel solution was degassed before adding TEMED and ammonium persulphate.

Separation gel was first poured into a gel mould (LKB Instruments Ltd.) to make a 16x16 cm and 0.3 mm thick slab. 15 minutes later the gel was polymerised and a layer of stacking gel was poured on top of it and a gel comb was fitted in carefully. After polymerisation of the stacking gel, it was clamped into the electrophoresis tank and the electrode reservoirs filled with one litre of electrode buffer composed of 25 mM Tris HCl, 192 mM glycine and 0.1% SDS. The pH of the Tris-glycine buffer was 8.3.

Sample buffer was made using 2% w/v SDS, 10% v/v glycerol, 5% v/v mercaptoethanol, 0.001% Bromophenol blue in 60 mM tris

HCl buffer. The concentration of LPS antigen was adjusted to 200 ug/ml and the concentration of surface protein antigen to 400 ug/ml of sample buffer.

Samples and reference standards were heated at 100°C for 5 minutes and allowed to cool. Then by removing the comb from the stacking gel ,50 ul of sample or standard was loaded into each slot in the following order. The second slot from each side was loaded with standard, the next one toward the middle with each test sample in duplicate.

Electrophoresis was carried out at 20 mA C.C. for 4 hours with a Atta Digipower Model SJ 1081 power pack (Atta Atto Corporation, Japan).

The gel was removed from the apparatus and divided into two sections. One of the sections was stained with 0.025% Coomassie brilliant blue for protein antigens (Fig. 6) and the other section was stained with silver stain for LPS by the method described below. The surface protein antigen was found to contain 2 major protein bands of molecular weights of 38000 and 41000 with a number of minor ones. No protein was detected in the LPS lanes (2 and 4).

Silver Staining for LPS: The gel was fixed in 200 ml of a solution of 25% isopropanol and 7% oleic acid overnight .Then it was left for 5 minutes in a freshly prepared solution of 1.05g of periodic acid, 4 ml of 25% isopropanol and 280 ul of 7% acetic acid 150 ml of distilled water.

The gel was washed six times, with 200 ml of distilled water for 15 minutes each time and stained in the silver staining solution for 10 minutes. Silver staining solution was prepared from 28 ml of 0.1 M NaOH, 1 ml of 29.4 % NH_4OH, 5ml of 20% silver nitrate in 115 ml of distilled water. After staining, the gel was washed four times, 10 minutes each time with 200 ml of distilled water and developed for 10-20 minutes

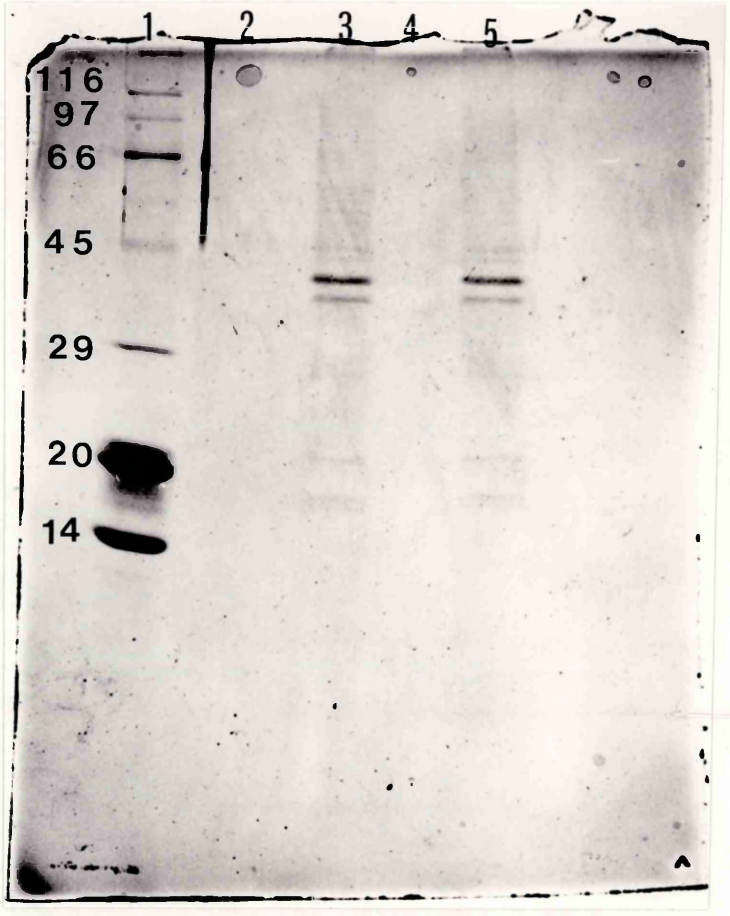


FIG. 6: Electrophoresis of LPS and surface protein antigens of S. typhimurium pt. 49 in SDS PAGE stained with 0.025% Coomassie Brilliant Blue R. Lane 1, molecular weight markers with sizes (x 1000), Lanes 2 and 4 LPS antigens, Lanes 3 and 5 surface protein antigens.

in 250 ml of developing solution (12 mg of citric acid and 0.125 ml of 37% formaldehyde in 250 ml of water) at 25°C.

The reaction was stopped by placing the gel in a solution of 5 ml of 7% acetic acid in 200 ml of distilled water for an hour. Finally the gel was washed with distilled water. The results are shown in Fig. 7. LPS antigen was shown to contain a number of LPS bands (Fig. 7, lanes 2 and 4) and protein antigen to contain a small amount only.

The results of electrophoresis of antigens on SDS PAGE showed pure LPS but surface protein antigen contained a small amount of LPS (Fig. 7). To evaluate the effect of this impurity in the performance of the test, M129 A plates were coated with pure LPS antigen and used in the subsequent ELISA procedures as controls for surface protein coated plates. The final absorbance reading was negligible (less than 0.1 for 1ug/ml of LPS) for a positive serum while those of protein coated plates were more than 1.5 for the same serum. When this serum was examined at the same dilution on a M129 B plate coated with LPS (1 ug/ml), the absorbance value was more than 1.5.

Validation of the ELISA was then continued by the following tests:

When SPF sera were used as a negative standard against anti-chicken IgA and IgM a high absorbance was noted. 100 ul of this serum was then mixed with 100 ul of either LPS or surface protein antigens, incubated at 37°C overnight, centrifuged and the precipitate discarded. The treated serum was examined again. The results are shown in Appendix 1 and discussed in Chapter 6.

Controls and standards were used to monitor the accuracy of the day to day experiments. Standards were a positive and a negative serum of known absorbance. For testing of egg samples an egg white or yolk examined previously was also included.

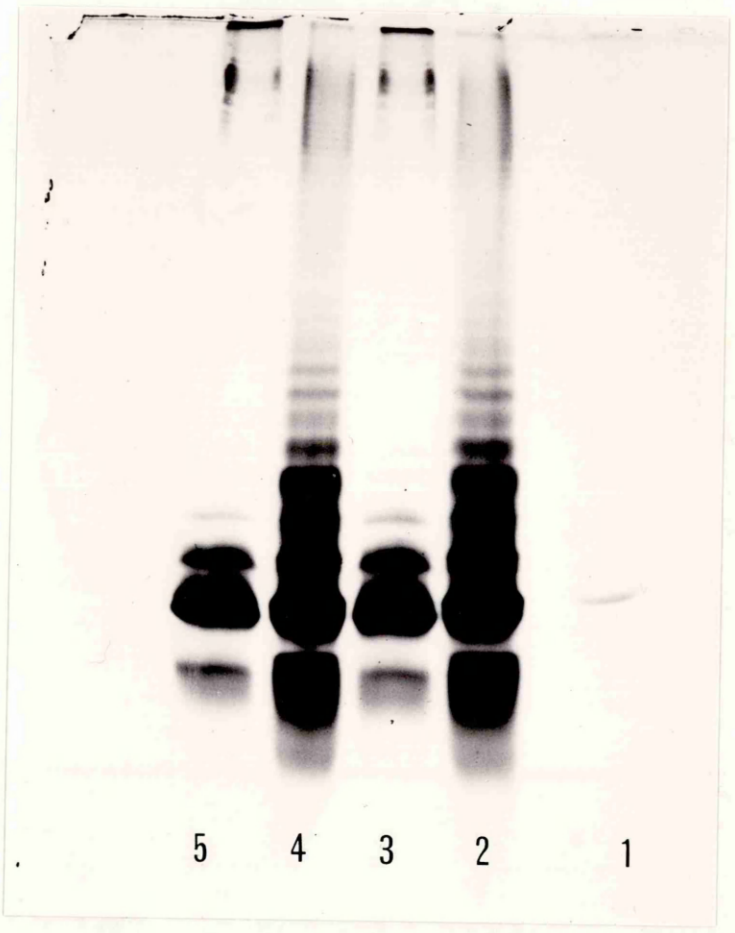


FIG. 7: Electrophoresis of LPS and surface protein antigens of S. typhimurium pt. 49 in SDS PAGE stained with silver stain. Lane 1, molecular weight markers (unstained), Lanes 2 and 4 LPS antigens, Lanes 3 and 5 surface protein antigens.

Controls included duplicate wells of two types, one with no sample and no antisera and the other with no sample only. The initial examinations showed zero absorbance for these wells.

d) Calculation of results and end points

Mean and standard deviations (SD) of the mean of the absorbance figures for each dilution were calculated for both positive and negative samples and a curve was plotted for each sample by using these figures against reciprocal dilutions of the samples. At high dilutions where two curves were approaching each other, the mean values plus five times the SD were monitored on both positive and negative curves and the last points where there was no overlap of two curves were chosen as end points.

The mean of the A_{490} plus 5 times the SD was usually around 0.05, but for higher accuracy, 0.1 was chosen as the end point. Before running test samples the exact values of A_{490} was determined for positive standards (reference) at a fixed dilution (A). This reference dilution was used on each plate in (duplicate) wells and its A_{490} (B) was used to standardise the results of different days'samples. There was usually no difference between A and B, but due to sensitivity of the test and difficulties in reproducing incubation times, temperatures and dilutions precisely, there were slight variations in absorbance of the reference samples in a few cases. In such cases, the sample readings were corrected using the following formula (Voller et al., 1979):

Corrected absorbance of sample= absorbance of sample x A/B

For example, with a value of 0.2 for serum IgG (A) and a reading of 0.25 for (B), a sample reading of 0.5 would be corrected to 0.5 x 0.2:0.25 = 0.4.

B) Immunoelectrophoresis

Gel bond film (FMC Bio Products, Maine, USA) or slides were covered with a layer of 1-1.5 mm thick one percent agarose (Pharmacia Fine Chemicals, Bucks, England.) in veronal buffer (pH 8.2). This buffer was made up by 1.4 g of barbitone (BDH Chemicals Ltd.), 5g of sodium barbitone, 0.01% thiomersal and 1 g of NaCl, in one litre of deionised water.

Gels were left on a levelling table and allowed to set for 30 minutes, then placed in a humid chamber at 4°C until used. Troughs 0.4 cm wide by 5 cm long and wells 0.3 cm in diameter were cut out of the agar with a cork borer and a scalpel. The distance between the troughs and wells was 0.4 cm. Bromothymol blue was used as an electrophoresis marker in one of the wells. The gel was placed in the central part of an electrophoresis tank containing veronal buffer pH 8.2. After loading samples in the wells, electrophoresis was carried out at 120 V for 4 hours at room temperature. Filter paper was used as an electrode wick and tap water was circulating around the electrophoresis chamber (Gelman Instrument Co.) to cool the gel. After the agar was removed from the troughs, 0.2 ml of antiserum at the optimal working dilution was placed in each trough and diffusion of the antiserum and antigens took place in a humid chamber at room temperature. After 24 hours' incubation the gels were washed for 24 hours in phosphate buffered saline (PBS) and for 6 hours in distilled water. They were then dried at room temperature or 37°C and stained with Coomassie brilliant blue at 0.025 percent.(Estrada 1986).

C) Double immunodiffusion test

1% (w/v) agar (Pharmacia Fine Chemicals, Bucks, England) was prepared in PBS pH 7.2 (Oxoid) containing 0.01 percent thiomersal. Plastic petridishes 3.5 cm in diameter or gel bond films 2.6 cm x 7.6 cm were covered with a 1.0-1.5 mm thick layer of 1% agar. The gels were left on a levelling table for

30 minutes to solidify. Using a cork borer 0.3 cm in diameter, wells were cut out in a circle 0.5 cm apart from each other and the central well. 15 microlitres of the sample to be tested were loaded in to the wells on the circle and the same quantity of antiserum was placed in the central well. Diffusion of antigens and antiserum was allowed to take place for 48 hours in a humid chamber at room temperature.

The gels were washed for 24 hours in PBS and 6 hours in distilled water, dried at room temperature or 37^{o}C and stained with Coomasie brilliant blue at 0.025%.

D) Agglutination test

Two types of agglutination test were carried out during this study.

1 - Slide agglutination test for the differentiation of Salmonella from other bacteria and determination of its specific serogroup as described in Section 1 of this Chapter .

2 - Rapid serum plate test: This test was performed by placing a drop of serum on a slide and mixing with a drop of freshly prepared suspension of S. typhimurium Pt. 49. After mixing, the slide was rotated gently and the test read within one minute. If agglutination happened within this time, the test was regarded as positive. If the reaction took two minutes to occur it was considered doubtful and no reaction or reaction after 2 minutes was considered as negative.

E) Indirect immunofluorescence on frozen sections

This study was carried out on frozen sections of the gut of the chicks infected with Salmonella typhimurium pt. 49. Specific antiserum raised (next section) against this bacterium in a rabbit was layered on frozen sections mounted on slides. The slides were incubated in the dark for 30 minutes

in a moist chamber at room temperature and washed twice, each time for 15 minutes in PBS pH 7.2 and once with distilled water for 5 minutes. Fluorescein conjugated anti-rabbit IgG (Miles Laboratories Ltd., Stoke Pages, Slough, U.K.) was applied to the sections and incubated at room temperature for 30 minutes. The slides were washed again as in the previous step and mounted with cover slips using 10% glycerol in PBS pH 9.0 and examined using a fluorescent microscope (Leitz, Wetzlar, West Germany). Controls were used and prepared under the same conditions, except that a negative rabbit serum was used instead of the rabbit anti Salmonella typhimurium pt. 49 antiserum.

F) Preparation of rabbit anti S. typhimurium pt. 49 antiserum

Salmonella typhimurium pt. 49 was subcultured from sloppy agar into nutrient broth, incubated overnight, in a shaking incubator at a speed of 140 rev/min at 37°C. 2 ml of this culture was diluted in 38 ml of fresh nutrient broth and incubated for a further 90 minutes. 0.5% formaldehyde solution was added to this culture and it was left overnight at 4°C. 0.2 ml of this culture was injected intravenously (IV) into the ear vein of a New Zealand White 12 week-old rabbit (B.S. and S Scotland LTD.) This antigen was freshly prepared every week and 0.3 ml, 0.5 ml injected by the IV route in weeks 1 and 2 after the first injection. A 0.5 ml dose was injected at weeks 3 and 4. The rabbit was bled from the ear vein 10 days after the first injection and the serum antibody titre was checked by serum agglutination. 10 days after last injection the rabbit was euthanised using sodium pentobarbitone (Euthatal^{R}, RMB Animal Health Ltd., Dagenham, England) and bled from the heart. The serum was separated, aliquoted and kept at -20°C.

3 - EXPERIMENTAL CHICKENS AND EGGS

All chickens and eggs used in this study originated from Wickham Laboratories (SPF Farms) Ltd., Winchester Road,

Wickham, Hants, England. All transport was by Red Star Parcel train.

A) Adult chickens

Adult chickens that were used in this study fell into 3 groups.

1 - SPF chickens: These were reared up to 16 weeks in the above mentioned farm and then transferred to an isolation unit in the Veterinary School for vaccination studies. Their maintenance and accommodation conditions are described in Chapter 4.

2 - Naturally infected chickens: These were grown from day old naturally infected chicks and during the whole period were kept in an isolated loose box in the Veterinary School.

3 - SPF hens: These were purchased from the above mentioned farm as donors of control sera, bile and oviductal secretions for the above two groups.

B) Day old chicks

a) The eggs and hatching conditions

All eggs were used in this study originated from Wickham Laboratories (SPF Farms) Ltd. by direct purchase or were obtained from experimental hens of the same stock.

All were set in incubators as soon as possible (within 10 days after laying) and monitored daily until hatching.

The incubators used were two Multihatch Automatic Incubators each with the capacity of 96 hens'eggs and one Polyhatch Automatic Incubator with the capacity of 42 hens'eggs. All were supplied by Brinsea Products Ltd., Banwell, Avon, England. The temperature, egg turning and humidity were

automatically controlled in these machines and each one was set according to the supplier's instruction manual. At day 19 of incubation the egg turner was switched off so as not to disturb the emerging chicks. The temperature was slightly decreased and the humidity increased at this time as outlined in the manufacturer's manual. When the hatch was complete, the chicks were removed from the incubators and each one was marked by putting a numbered wing tag into the wing web. The number on the wing tag was used to identify the chicks during the experiment.

Every chick was individually swabbed before transfer to the experimental accommodation.

All unhatched eggs and these swabs were monitored for the presence of Salmonella as described in Section 1 of this Chapter.

Incubators were thoroughly cleaned and washed with a formaldehyde and glutaraldehyde disinfectant solution (Tegodor, TH. Goldschmidt Ltd, Eastcote, Middlesex) after each use. They were also fumigated by putting a container of formaldehyde solution (BDH) inside each incubator prior to incubation of the eggs. Light fumigation was used after setting the eggs and during incubation.

b) Maintenance and accommodation

The day old chicks used throughout this study were hatched in the animal house of Department of Veterinary Pathology as described above. These chicks were used in colonisation and challenge studies as explained in Chapters 3, 4 and 5.

After hatching these chicks were transferred to an isolated loose box, where the experiments were carried out. They were divided into different groups according to the experimental plan and each group was accommodated in a pen.

Each pen was constructed of a wire mesh floor over a dropping tray and a metal cage 30 cm in height formed the walls of each pen. The cage was open top and bottom. An infrared Ali-Brooder (Brinsea products Ltd., Banwell, Avon, England) was placed in each pen to provide warmth and light for the chicks in each pen. The accommodation was warmed up prior to the commencement of each experiment and kept warm to avoid any cold stress on the chicks. Water was available from a dripping nipple connected to a bottle and was mounted on the wall inside each pen and the height was adjusted to the size of the chick. Feed was supplied by a gravity type feeder and one or two were placed in each pen according to the numbers of chicks. A photograph of an assembled pen holding some experimental chicks is shown in Fig. 8.

These pens were used for chicks from day old up to 4 weeks old and if the experiment was running for longer time, the chicks were transferred to a loose box on wood shaving litter as described in Chapter 3.

c) Feed and water

Feed and water was available ad-lib. 5 M Rad irradiated starter pellets (supplied by Labsure, Lavender Mill, Manea, Cambridgeshire, England) were used up to the age of 3-4 weeks. After this time it was replaced by grower pellets supplied by the same manufacturer. The declared composition of these feeds is shown in Appendix 2.

d) Inoculation of day old chicks

A 16 hour nutrient broth culture of S. typhimurium pt. 49 was prepared and diluted as described in Section 1 of this Chapter. 0.5 ml of this inoculum was placed in the crop of each chick using a sterile single use Blue Line Umblical Cannula (8 FG)(Portex Ltd., Hythe, Kent, England) that was attached to a sterile syringe.

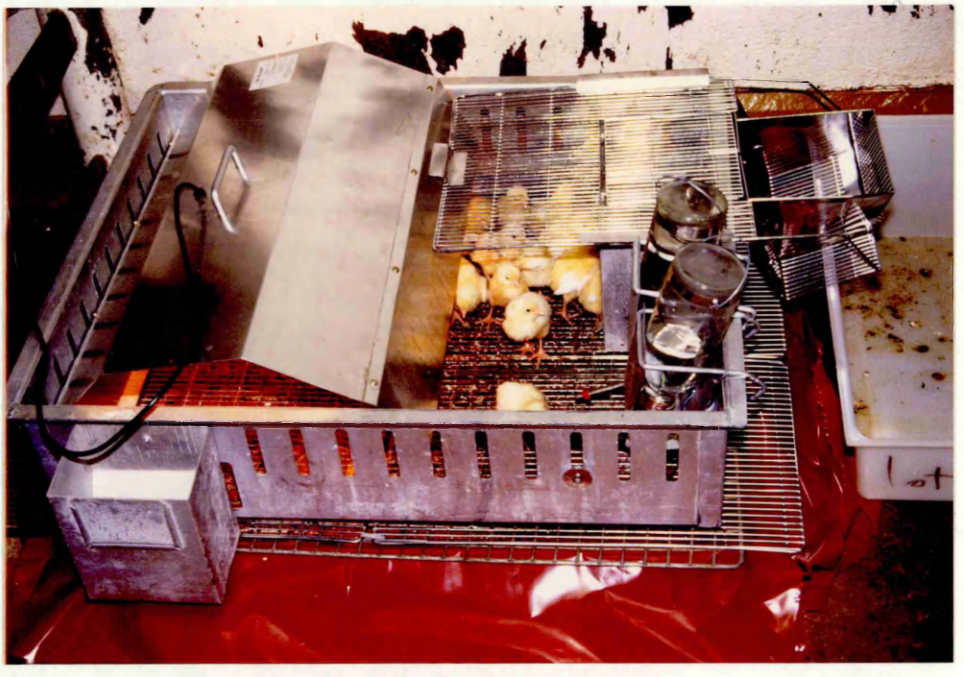


FIG. 8: Photograph of a pen housing young chicks in challenge studies.

The chick was held in one hand and with the other the cannula was inserted gently through the mouth and oesophagus into the crop. Then by pressing the plunger of the syringe the required amount was inoculated and the cannula withdrawn. In some cases inoculum was spilled from the corners of the beak.

4 - DIAGNOSTIC PROCEDURES

The following methods were used frequently during this study for diagnostic purposes:

A) Clinical examination and monitoring

All birds were closely watched during each experiment. Any abnormal signs in their behaviour, gait, respiration, feed and water consumption, attitude toward their environment and other birds, faecal passage and its consistency were carefully noted and recorded. The adult birds were examined for the presence of external parasites, tumours, abscesses, skin changes, beak, claw, comb, feather and abnormalities of bodily condition.

B) Samples and sampling methods

The samples used for bacteriological studies and their preparation are explained in section 1 of this Chapter. The rest are as follow:

a) Blood samples and serum separation

Blood samples were obtained either by venepuncture or by cardiac puncture under terminal anaesthesia (Zander 1984).

(i) Bleeding from vein

Whenever it was necessary to keep the birds alive after bleeding, the blood specimens were taken from the main (brachial) wing vein. The vein was exposed to view by plucking

a few feathers from the area around the vein and the skin in the area was dampened with 70% alcohol. While another person was holding the bird gently and firmly a 20 mm, 20 gauge needle was used to bleed the bird. About 1-2 ml blood was collected from each bird and was left in the 5ml syringe to clot at room temperature. After an hour the clot was removed into a universal and left at 4°C overnight. Any haematomas on the wing were recorded.

(ii) Bleeding from heart

This was carried out under terminal anaesthesia. The bird was held on its back with the keel up. Using a forefinger, a 2 inch long and 20 gauge needle was guided through the thoracic inlet toward the heart and the sample was obtained. Immediately the blood was poured into a universal and left at room temperature to clot. After an hour the clot was ringed and placed at 4°C overnight.

(iii) Separation of sera

Clots that were left overnight at 4°C had contracted and sera were separated and centrifuged at 2000 g for 20 minutes at 4°C to remove the cells. Then the serum was stored at -20°C until used. In some cases when the clot had not been yielded enough serum, the clot itself was first centrifuged at 2500 g for 30 minutes at 4°C and then serum was separated (Johnstone and Thorpe, 1982).

b) Egg collection and maintenance

Eggs were used for:
-Incubation to produce chicks.
-ELISA to determine to determine the immunoglobulins titre in the yolk and white.
-Isolation and purification of the immunoglobulins.

All eggs were collected daily, cleaned and stored at +4^{o}C until used. The eggs for incubation were used within 10 days of collection but those for other purposes were sometime stored for longer.

C) Euthanasia

All live chickens were killed by the intraperitoneal or intracardiac injection of sodium pentobarbitone (Euthatal, RMB Animal Health Ltd., Dagenham, England).

D) Post mortem examination

a) Gross examination

The feather or fluff was dampened with disinfectant solution and the bird was laid on its back. By cutting the skin between the legs and the abdomen and bending them towards the back, the head of the femur was dislocated from the acetabular attachment so that the legs were lying flat on the table. Then by cutting the abdominal skin and reflecting it forward and backward the entire ventral aspect of the body was exposed. Small chicks were laid on a wax tray and fixed by pins or needles to the tray.

The abdominal cavity was opened aseptically and visceral organs revealed. Any gross lesions on the organs inside the thoracic and abdominal cavity including air sacs and yolk sac (in young chicks) were noted and recorded and some of them photographed Samples were taken for bacteriological, immunological and histological studies.

b) Histological procedures

Samples were taken from the intestine and caeca for studying the adhesion of Salmonella to the mucosal surface of the gut in SPF infected chicks.

The samples for light microscopy were fixed in 10% neutral buffered formal saline for at least 48 hours, then embedded in paraffin wax and sections were cut at 4-6 um and stained with haemotoxylin and eosin (H&E) and examined by light microscopy. Section preparation and staining was carried out by the staff of the histopathology laboratory of the Department.

Sections were also prepared for electron microscopy and indirect immunofluorescence test and studied. Carnoys fixative was used for immunofluorescent sections and Karnovsky fixative for electron microscopic sections.

D) Precautionary measures for prevention of spread of Salmonella

Due to the potential risk from Salmonella, strict safety measures were carried out during the experiments. Infected birds were attended only by authorised persons, wearing overalls and boots. At the entrance of the accommodation a disinfectant tray was placed and every day the disinfectant solution was changed. Sodium di-Chloroisocyanurate disinfectant tablets (Presept, Surgikos Ltd., Livingston, Scotland.) were used. All of the equipment and pens were thoroughly cleaned and washed with Tegodor solution after the end of each experiment. All equipment necessary for running the experiment was placed inside the accommodation boxes and fumigated with 0.3% Tegodor solution using an Atomist fogging machine model No. 1021X Atomist (Th. Goldschmidt Ltd., Eastcote, Middlesex). Washing and disinfectant facilities were available in the boxes.

The accommodation was made bird and rodent proof to prevent the entrance of wild birds and rodents. Insecticide was used to kill any insect that entered the boxes. Plastic disposable gloves were used to handle the chickens and infected equipment inside the boxes. All used and disposable materials and carcasses were incinerated.

CHAPTER 3

STUDY OF NATURAL IMMUNITY

1 - INTRODUCTION

In the first part of this series of studies the capability of the strain of S. typhimurium pt. 49 to colonise the gut of the chicken was confirmed experimentally (Experiment 1). Naturally infected chicks were reared until adulthood to provide hens and cocks for immune egg production (Experiment 2). The presence of specific immunoglobulin in their sera, eggs, bile and oviductal secretion was determined using the ELISA described in Chapter 2, Section 2. Chicks hatched from immune eggs were challenged using the same organism to evaluate the role of naturally transferred specific immunity in the protection of young chicks (Experiments 3 and 4).

2 - INITIAL COLONISATION STUDY (EXPERIMENT 1)

A) Materials and methods

50 SPF eggs were purchased from Wickham SPF farms and incubated as described in Chapter 2, Section 3. 44 chicks hatched out of 50 eggs, 3 eggs were infertile and 3 did not hatch. 2 of the chicks which hatched were not included due to weakness and bruising of the legs and the rest of them were divided into two groups of 21 chicks at random. All were identified by wing tags and transferred to the accommodation described in Chapter 2, Section 3. Both groups were kept in the same room but in separate pens. On day zero one group was inoculated individually with $1x10^3$ CFU of 16 hour broth culture as described in Chapter 2, Section 3. Feed and water was available ad-lib to both groups after inoculation of the chicks in the experimental group.

Any approach was first made to the control (uninoculated

group) wearing disposable gloves and apron on top of a lab coat. When moving from one group to another the gloves were changed.

Unhatched eggs and culled chicks were cultured for Salmonella. The progress of the disease was checked daily by monitoring the clinical signs. Cloacal swabs were taken from all of the chicks before inoculation and then daily for up to 11 days post inoculation to monitor Salmonella excretion. The extent of colonisation was monitored by counting Salmonella from fresh droppings as well as from the gut contents of chicks killed on days 5 and 11 after infection. The pathogenesis of the infection was assessed by preparing histological sections from the intestine and examining them by transmitted light, indirect immunoflourescence and electron microscopy as well as by culturing the internal organs and looking for gross lesions.

B) Results

a) Clinical signs

Mild growth depression, diarrhoea, decrease in food consumption, pasting of the vent, drooping of the wings and ruffled feathers were observed during the period of the experiment. The orally inoculated chicks were more retarded in growth compared with the control group that acquired the infection naturally.

There was 100% morbidity in both experimentally and naturally infected groups. Mortality due to salmonellosis was 0.0 and 9.5% (2 chicks out of 21) in naturally and experimentally infected groups respectively.

b) Gross lesions

After death or killing of the chicks by Euthatal, post mortem examination was carried out as described in Section 4 of

Chapter 2. The following gross lesions were noted in different chicks died or were killed between days 5 and 11 post infection.

Fibropurulent pericarditis and perihepatitis and adhesion between visceral organs were the most prominent lesions especially in birds which died (Fig. 9). Other lesions included emaciation, dehydration, coagulated yolk (only in dead chicks) and enteritis. Caecal cores or inflamed caeca containing frothy contents were also observed in some of the chicks. Mild hydropericardium and congestion of the liver were seen in those chicks that were killed by Euthatal.

c) Histological monitoring of the pathogenesis of Salmonella infection

Examination of histological sections of the small intestine was carried out. Gram-negative bacilli and coccobacilli were seen in the lumen of the small intestine and between the villi. In some cases bacteria were seen apparently adhering to the villous epithelium (Fig. 10). Neutrophils were present in the lamina propria but it was not clear whether villous stunting was present because of the plane of section of the intestine.

Organisms were seen in the lumen of the caecum and adjacent to the mucosal epithelial surface in small numbers. Adhesion between bacteria and this luminal surface was not clear. In a few sites inflammatory cells were seen in the mucosal epithelium (Fig. 11) and in one place a micro-colony of organisms was seen within a cell (Fig. 12). The caecal lamina propria was thickened and contained plasma cells, neutrophils and distended capillaries. The bacteria seen were Gram-negative but the Gram-stained sections were less distinct than those stained by H&E and are not illustrated.

The livers of the chicks contained a variety of lesions.

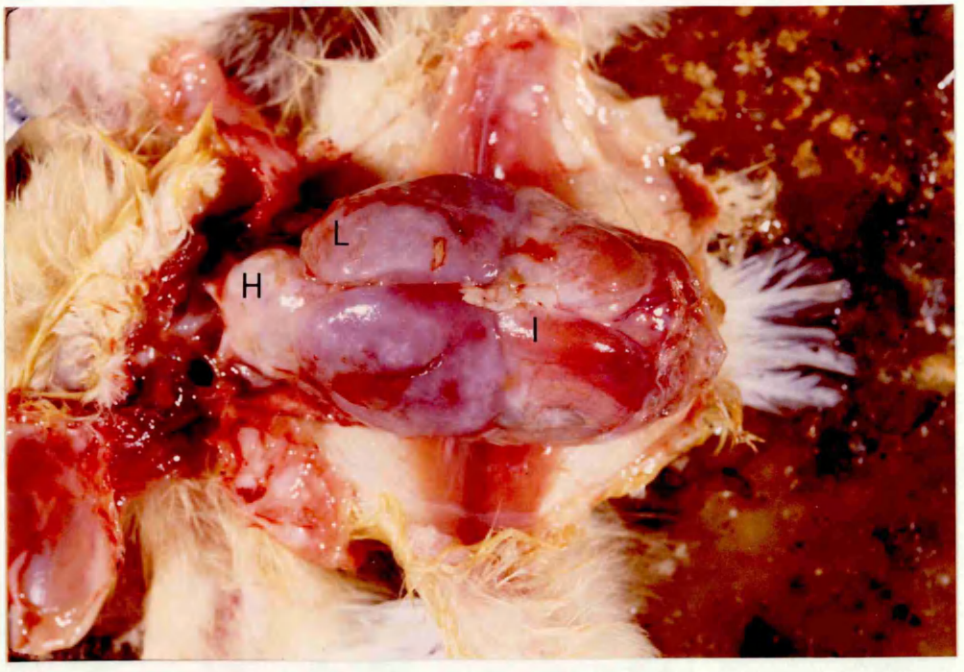


FIG. 9: Gross lesions in chick 807 infected with S. typhimurium pt. 49 (died on day 8 post infection). Note the pericarditis and perihepatitis.
L: Liver, H:Heart, I: Intestine.

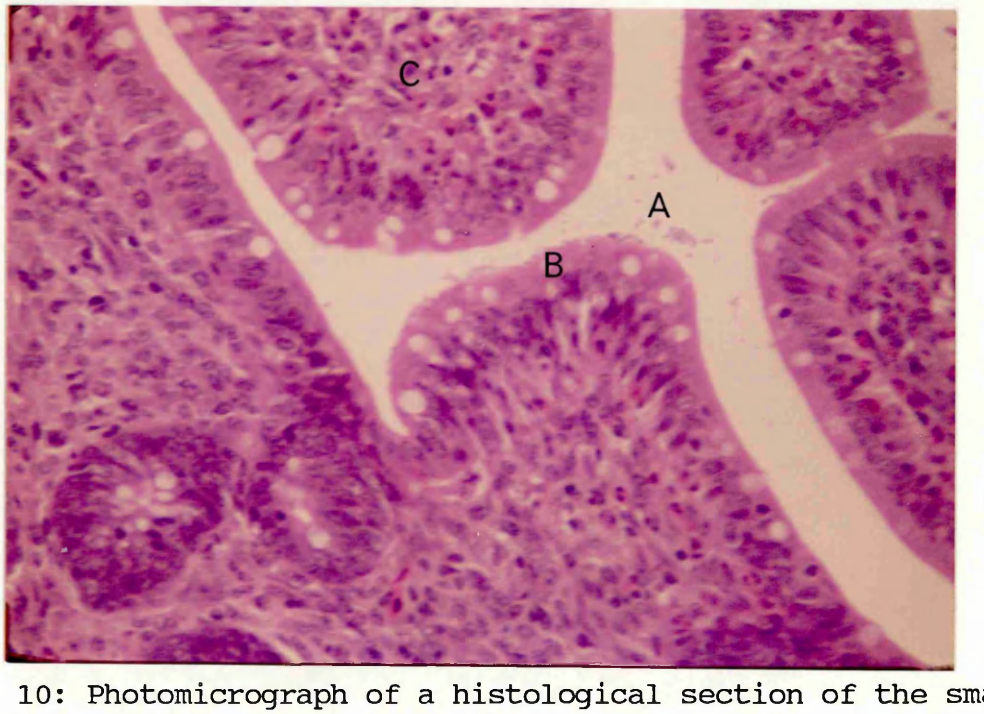


FIG. 10: Photomicrograph of a histological section of the small intestine of chick 817 killed on day 5 post infection. Bacteria were present (A) between the villi and apparently adhering to the brush borders (B). Inflammation of the villous core is present (C). H&E x 320

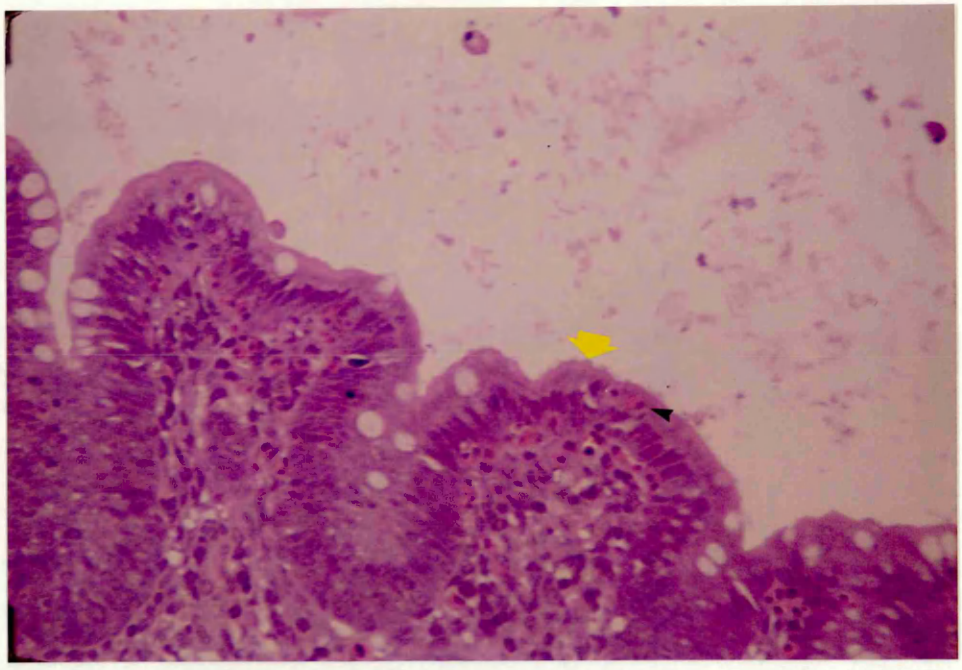


FIG. 11: Photomicrograph of a histological section of the caecum of infected chick 820 killed on day 5 post infection. Bacteria were present in the lumen adjacent to the mucosal epithelial surface (yellow arrow). Inflammatory cells can be seen in the mucosal epithelium (black arrow). H&E x 320

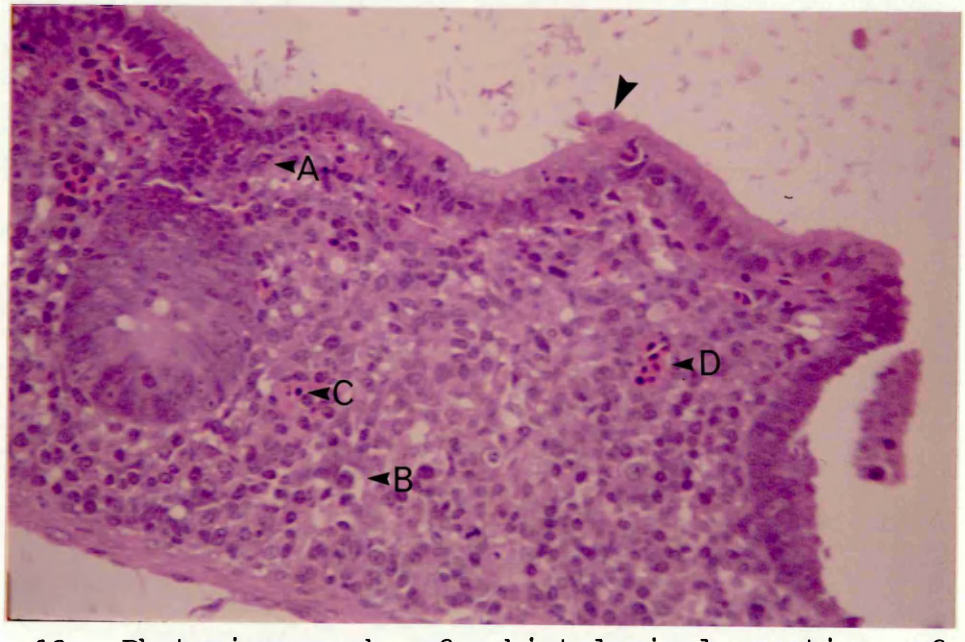


FIG. 12: Photomicrograph of a histological section of the caecum of infected chick 817 killed on day 5 post infection. A micro-colony of the organism (black arrow) is present in the mucosal epithelium, and thickened lamina propria (A) with plasma cells (B), Neutrophils (C) and distended capillaries (D) can be seen. H&E x 320

In some (Fig. 13) small numbers of bacteria were seen in the capillaries. In others micro-colonies of organism could be seen (Fig. 14). These proved to be Gram-negative in gram-stained sections. In others there were scattered necrotic foci some of which were heavily infiltrated with inflammatory cells and typical of those of salmonellosis (Fig. 15). Indirect immunofluorescent studies confirmed the presence of Salmonellae on the epithelial surface and inside the epithelial cells and within the capillaries of the lamina propria of the small intestine.

The presence of organisms resembling Salmonella in the caecum and intestine were noted in E.M. studies. They were present in clumps and groups in the lumen (Fig. 16) but no organisms were seen attached to the intestinal epithelium or within cells in spite of extensive search. The micro-villi of the caecal epithelial cells were damaged in many cases (Fig. 16).

d) Salmonella isolation

No Salmonella were isolated from the infertile and unhatched eggs or the culled chicks. The results of daily monitoring by cloacal swabbing are shown in Table 3. All of the chicks in the inoculated group were positive every day starting from 24 hours after infection until day 11 when this experiment ended. The chicks from the control group acquired the infection from day 2 of the experiment and excreted the organism continuously as described below. 10^9 CFU of Salmonella per gram of fresh droppings were counted from both groups (Table 3).

The guts of all five chicks killed on day 5 post inoculation were colonised and 4 out 5 showed systemic infection (Tables 4 and 5). Intestinal colonisation was found in all 14 chicks killed on day 11 after inoculation and 10 were systemically infected. Higher levels of infection were found in liver and spleen than in heart and bile (Tables 5 and 6).

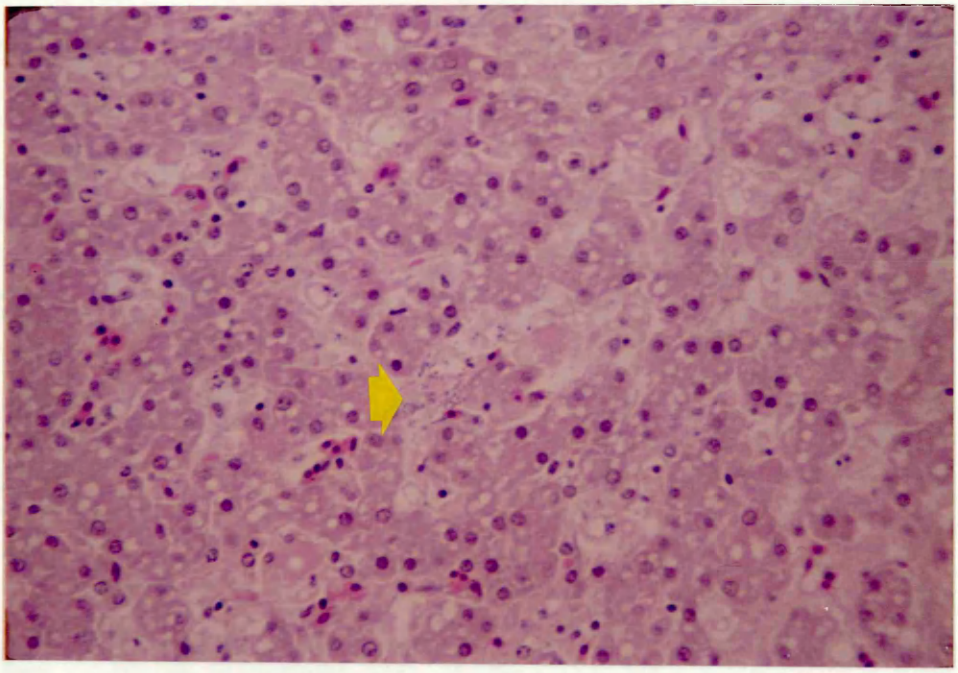


FIG. 13: Photomicrograph of a histological section of liver of infected chick 818 killed on day 11 post infection. Bacteria (arrow) can be seen in a capillary. H&E x 320

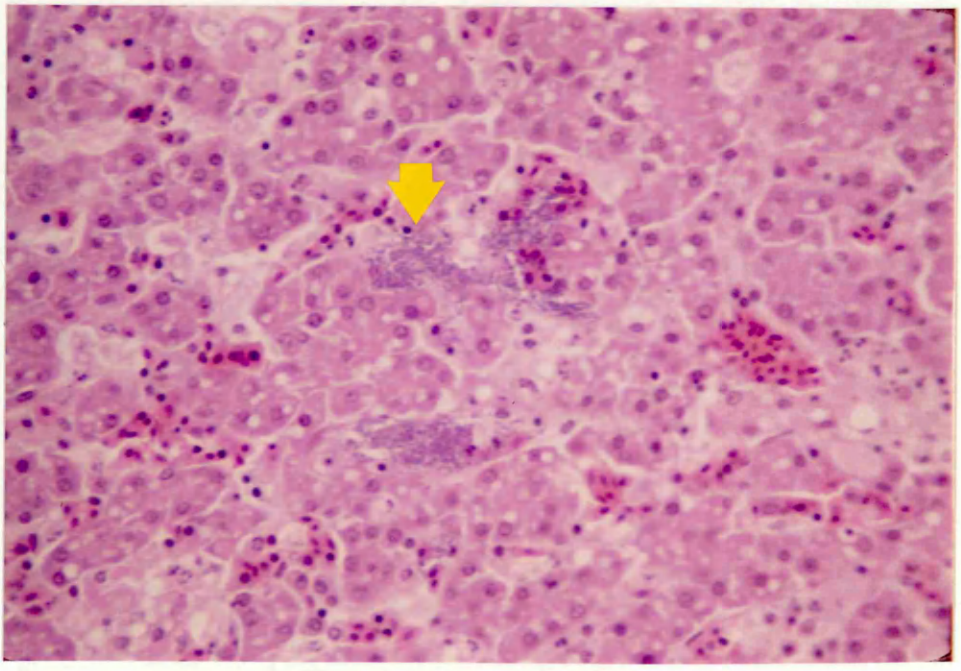


FIG. 14: Photomicrograph of a histological section of liver of infected chick 808 killed on day 5 post infection. Micro-colonies of the bacteria can be seen (arrow). H&E x 320

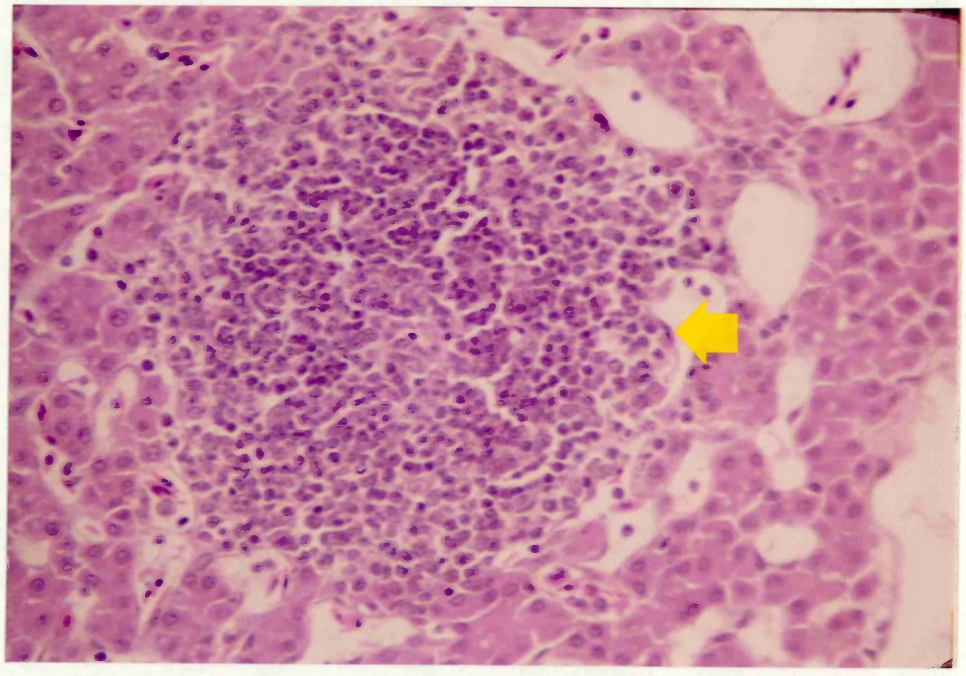


FIG. 15: Photomicrograph of a histological section of liver of infected chick 810 killed on day 11 post infection. A necrotic focus (arrow) with a heavy infiltration of inflammatory cells is present. H&E x 320

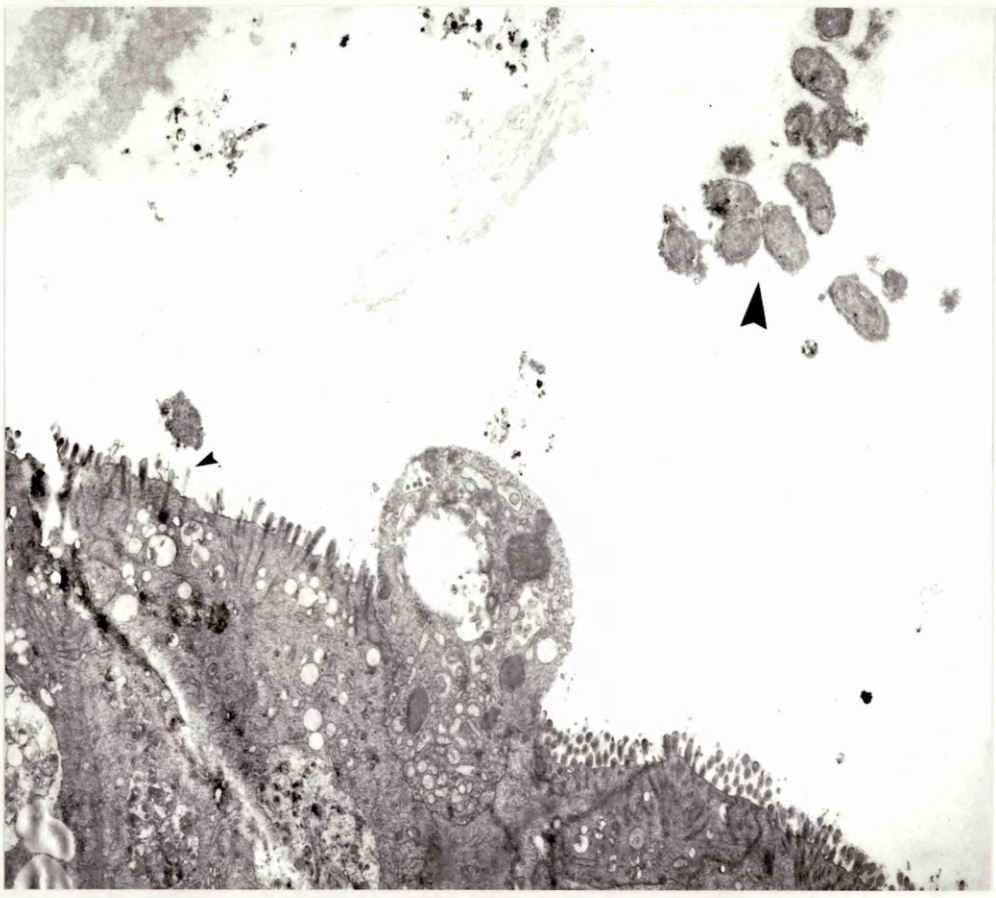


FIG. 16: An electron micrograph of the caecum of infected chick 814 killed on day 5 post infection. Bacteria with the morphology of Salmonella (big arrow) and damaged micro-villi (small arrow) are present.
x 8800

Table 3

Salmonella isolation from cloacal swabs (qualitative) and pooled fresh droppings (quantitative) during Experiment 1.

Days of experiment	Qualitative No.positives:total swabbed		Quantitative CFU/g	
	Inoculated	Control	Inoculated	Control
0.0	0:21	0:21	0.0	0.0
1	21:21	0:21	2.75×10^9	0.0
2	21:21	10:21	2.25×10^9	$<10^2$
3	21:21	20:21	1.5×10^9	1.0×10^9
4	21:21	21:21	5.0×10^9	2.0×10^9
5	21:21	21:21	3.5×10^8	1.5×10^9
6	16:16*	21:21	2.5×10^9	2.5×10^9
7	16:16	21:21	2.0×10^9	2.5×10^9
8	15:15**	21:21	1.75×10^9	2.0×10^9
9	15:15	21:21	2.5×10^9	1.75×10^9
10	14:14**	21:21	7.5×10^8	2.0×10^9
11	14:14	21:21	3.0×10^9	3.5×10^9

* = 5 chicks killed on day 5.

** = 1 chick died on each day.

Table 4

Gut colonisation and systemic infection in the orally infected SPF chicks after killing or death during Experiment 1.

Days after infection	No. of chicks examined	No. systemically infected	No. with gut colonisation
5	5 (killed)	4	5
8	1 (died)	1	1
10	1 (died)	1	1
11	14 (killed)	10	14
Total	21	16	21
%	100	76.19	100

Table 5

Salmonella isolation from different organs of SPF chicks orally inoculated with S. typhimurium and killed on day 5 after infection.

organ cultured	Direct culture (1)	After tetrathionate enrichment (2)	Quantitative culture (3) CFU/g
Heart	0:5*	2:5	N.D.
Liver	0:5	4:5	N.D.
Spleen	0:5	4:5	N.D.
Bile	0:2	0:2	N.D.
Crop	1:1	1:1	N.D.
Duodenum	0:2	0:2	0.0
Jejunum	2:2	2:2	1.75×10^6
Ileum	2:2	2:2	5.0×10^7
Caecum	5:5	5:5	3.0×10^9
Gizzard	1:1	1:1	N.D.
Proventriculus	1:1	1:1	N.D.
Rectum	1:1	1:1	3.0×10^9

(1) = Samples cultured directly on sheep blood, MacConkey and BG agar without enrichment. (2) = Subcultured onto DCA, SS and BG agar. (3) = Only one chick used.

* = No. of positives:Total No. cultured. N.D. = Not done.

Table 6

Salmonella isolation from the organs of 14 chicks killed on day 11 after infection in Experiment 1.

Organ cultured	Direct culture (1)	After tetrathionate enrichment (2)	Quantitative culture (3) CFU/g
Heart	1:14*	5:14	N.D.
Liver	2:14	10:14	N.D.
Crop	7:7	7:7	2.0×10^6
Jejunum	2:3	2:3	$>10^3$**
Ileum	2:2	2:2	1×10^6
Caecum	13:14	14:14	1.5×10^9

(1) = Samples cultured directly on sheep blood, MacConkey and BG agar without enrichment.

(2) = Subcultured onto DCA, SS and BG agar.

(3) = Data chosen from one chick.

* = No. of positives:Total No. cultured.

** = Due to the overgrowth of the coliform bacteria it was not possible to count the colonies exactly.

N.D. = Not done.

Salmonella was isolated from most parts of the gut (Tables 5 and 6), but the highest numbers (>10^9 CFU/g) were demonstrated in the caecal contents (Tables 5 and 6, Figs. 17, 18 and 19).

3 - DEMONSTRATION OF ANTIBODY IN HENS AND PRODUCTION OF IMMUNE EGGS (EXPERIMENT 2)

A) Material and methods

a) General maintenance conditions

All of the chicks in the control group of the Experiment 1 that were naturally infected were transferred to another isolated box which was set up for rearing of these chicks to adulthood.

The accommodation was an isolated loose box, 1.5 m wide, 2m long and 2.5 m high and was used during the growing and laying period. Wood shavings were used as litter and frequently changed for a new formaldehyde fumigated supply of the same material.

Feed and water were available ad-lib. The starter and growing pellets used were those described in Chapter 2, Section 3. At the age of 22 weeks the growing diet was replaced by 5 M Rad irradiated adult chicken pellets from the same source. Feed was supplied daily in a trough 10 cm deep, 8 cm wide and 40 cm long. A plastic gravity waterer with a reservoir volume of 10 litres was used for water supply. The height of the feeder and the waterer were adjusted to the size of the chickens.

Infra-red light bulbs were used for light and heat supply. The light was controlled to produce artificial day and night. 8 hours of daylight was provided until the chickens were 20 weeks old and was then increased 1/2-1 hour weekly using a timer switch to 16 hours at the age of 32 weeks, and then kept constant until the end of the experiment.

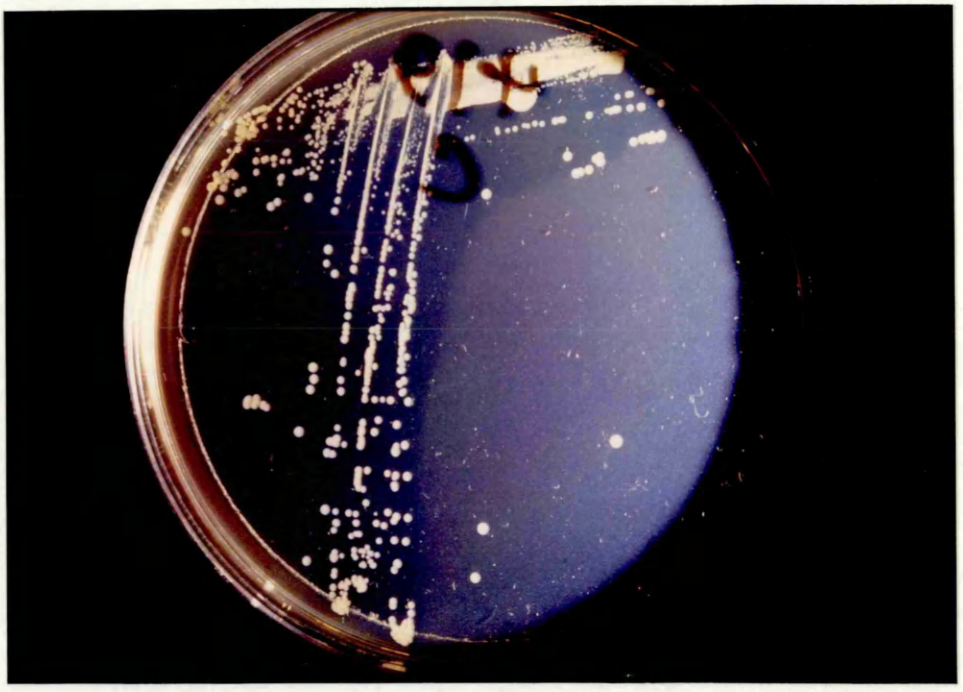


FIG. 17: S. typhimurium pt. 49 colonies isolated from caecal contents of infected chick 419 killed on day 7 post infection. Overnight culture of a sample pre enriched in tetrathionate broth on DCA agar.

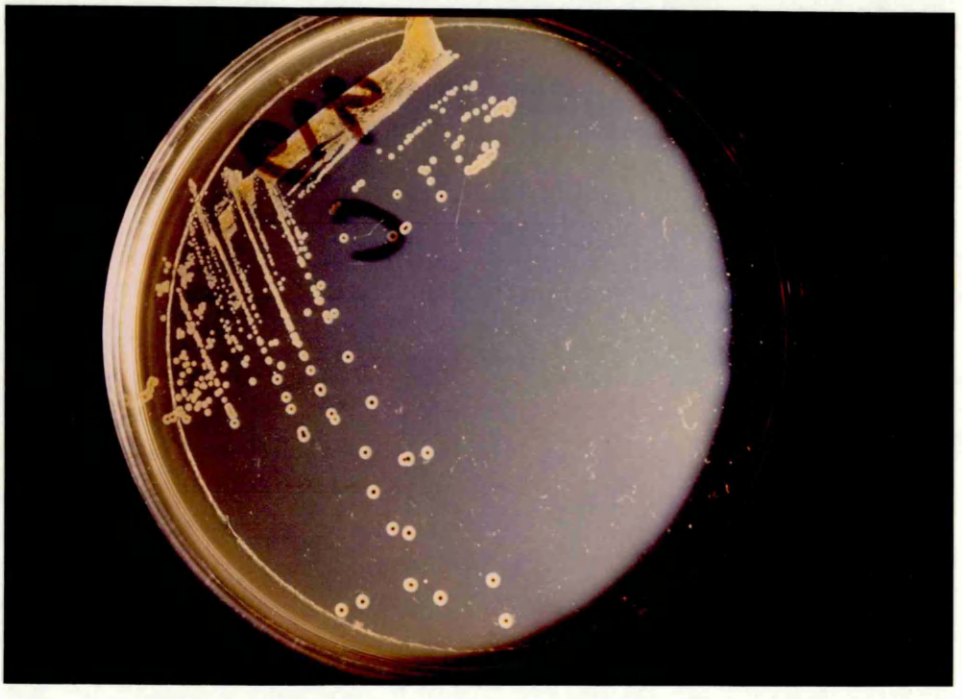


FIG. 18: S. typhimurium pt. 49 colonies isolated from caecal contents of infected chick 419 killed on day 7 post infection. Overnight culture of a sample pre enriched in tetrathionate broth on SS agar.

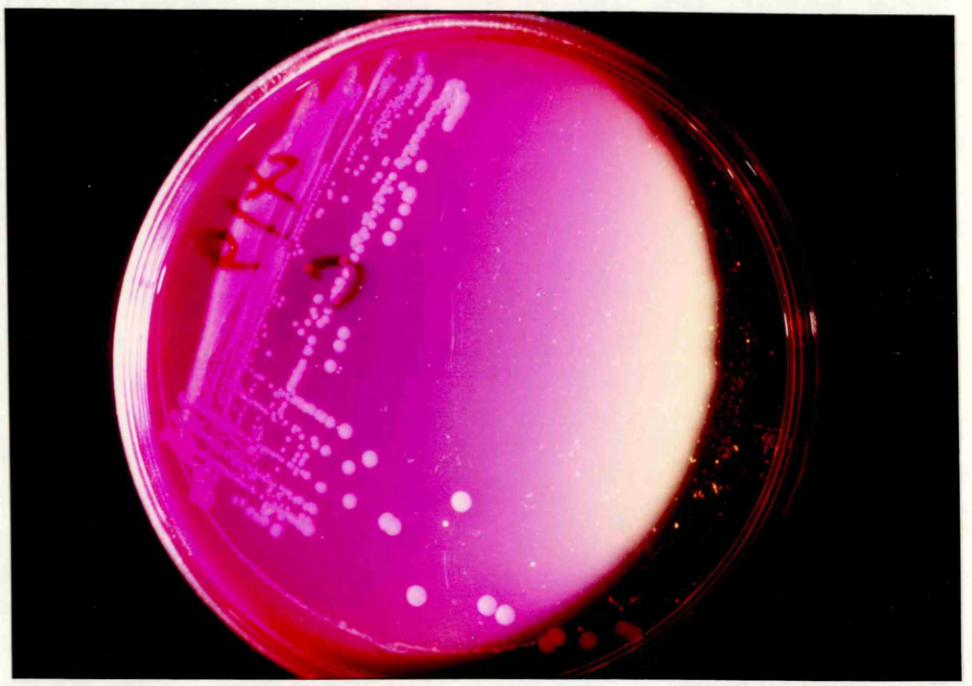


FIG. 19: S. typhimurium pt. 49 colonies isolated from caecal contents of infected chick 419 killed on day 7 post infection. Overnight culture of a sample pre enriched in tetrathionate broth on BG agar.

b) Monitoring the status of infection

Cloacal swabs, eggs, pooled faeces and litter and at the end of the experiment the internal organs and gut contents were cultured for Salmonella isolation during the period of Experiment 2 at the intervals shown in Tables 7 and 8. Feed and water were examined too. The samples were cultured as described in Chapter 2, Section 1.

c) Measuring specific immunity

Using the ELISA described in Chapter 2, Section 2, the levels of IgA, IgM and IgG against LPS and surface protein antigens in serum, bile and oviductal secretions were determined. The same samples from SPF hens were also used for comparative studies described in this Chapter and in Chapter 4.

Six SPF hens were purchased from Wickham SPF farms and transferred to the Veterinary School for sampling. The collection of bile and oviduct secretions after killing of the chickens was described in Chapter 2.

All serum samples collected in this study were screened by the agglutination test as described in Chapter 2.

B) Results

a) General health conditions

One of the 21 naturally infected chicks died due to intestinal haemorrhage, 9 cocks were killed and 2 cocks and 9 hens were reared for egg production. Apart from the mild clinical signs in early days after infection already described, no other signs of clinical abnormality were seen during the experiment. The beaks of all chickens were trimmed to prevent cannibalism. Toe-nails (claws) of the cocks were also trimmed to prevent the injury of the hen's back at mating.

Table 7

Salmonella isolation by cloacal swab culture during Experiment 2.

Age in days	Direct culture (1)	After tetrathionate enrichment (2)
11	21:21	21:21
19	19:21	21:21
33	0:20*	16:20
81	0:20	3:20
135	0:12**	1:12
186	0:11***	1:11

(1) = Samples were cultured directly on sheep blood, MacConkey and BG agar without enrichment.

(2) = Subcultured onto DCA, SS and BG.

* = One chick died at the age of 21 days.

** = 8 cocks were killed at 88 days of age.

*** = One more cock killed at 135 days of age.

Table 8

Salmonella isolation from fresh pooled faeces and litter of naturally infected chickens during Experiment 2.

Age in days	Nature of samples	Qualitative culture*		Quantitative culture CFU/g
		Direct	After enrichment	
19	Faeces	positive	positive	2.5×10^6
	Litter	N.D.	positive	N.D.
33	Faeces	negative	positive	$>10^5$
	Litter	N.D.	positive	N.D.
135	Faeces	negative	positive	N.D.
	Litter	N.D.	negative	N.D.
214	Faeces	negative	negative	N.D.
	Litter	N.D.	negative	N.D.
224	faeces	negative	negative	N.D.
	Litter	N.D.	negative	N.D.

* = The samples were directly cultured onto sheep blood, MacConkey and BG agar in parallel to tetrathionate enrichment which was subcultured onto DCA, SS and BG agar.
N.D. = Not done.

b) Salmonella isolation

Salmonella shedding is shown in Tables 7 and 8. As shown in Table 3 (Experiment 1) these chicks were continuously excreting the organism in their faeces at the level of 10^9 CFU/g up to day eleven, the day the colonisation studies ended. Salmonella shedding decreased in quantity as the chicks grew older (Table 8). All chicks were shedding the organism at the age of 19 days, 16/20 at the age 33 days and only 1 hen at the time of commencement of lay (Table 7). Salmonella was not isolated from any of the hens or cocks at the end of the experiment (Table 9). Cultures of the litter and fresh faecal samples failed to reveal any Salmonella on days 214 and 224 of the experiment (Table 8).

No Salmonella were isolated from the feed, water and unincubated eggs of naturally infected chickens. No Salmonella were isolated on culture of the caecal contents of the culled SPF hens.

c) Egg laying

Egg laying commenced at the age of 184 days with one egg. At the beginning the eggs were small (less than 55 g) but, gradually, larger eggs were produced. 3 weeks later the eggs were in the range of 55 to 65 g. The percentage of laying increased sharply and there were nine eggs (from 9 hens) on day 219 (35 days after laying commenced). Eggs were collected daily, cleaned and stored as described in Chapter 2.

d) Agglutination test

Serum agglutination tests were found to be easily interpretable (Fig. 20) in spite of the fatty serum. The results of the agglutination tests carried out on the screening samples are shown in Table 10. At the point of lay the sera of 2 hens were positive, 2 doubtful and the rest including the

Table 9

Salmonella isolation from different organs of naturally infected chickens killed during and at the end of Experiment 2.

No. of chickens	Age (days)	Organs cultured	Direct culture (1)	After enrichment (2)
1 (died)	21	Heart	0:1*	0:1
		Liver	0:1	0:1
		Jejunum	1:1	1:1
		Ileum	1:1	1:1
		Caecum	1:1	1:1
8 (killed)	88	Caecum	0:8	1:8
1 (killed)	135	Caecum	0:1	0:1
11 (killed)	257	Heart	0:11	0:11
	&	Liver	0:11	0:11
	307**	Spleen	0:11	0:11
		Bile	0:11	0:11
		Jejunum	0:11	0:11
		Ileum	0:11	0:11
		Caecum	0:11	0:11

(1) = The samples were directly cultured on sheep blood, MacConkey and BG agar without enrichment.

(2) = The samples were cultured into tetrathionate broth and then subcultured onto DCA, SS and BG agar.

* = No. of positives:Total No. cultured.

** = 4 hens and one cock were killed at the age of 257 day and the rest (5 hens and one cock) at the age of 307 day.

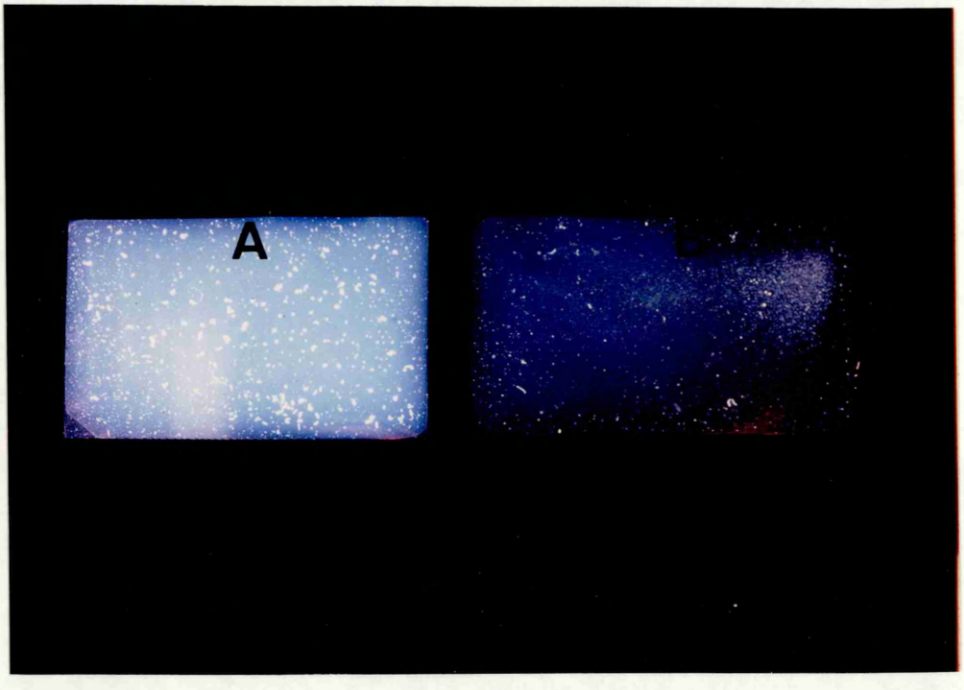


FIG. 20: Photograph of an agglutination test carried out on the sera of an infected (A) and a SPF chicken (B).

Table 10

Results of agglutination tests on the sera from naturally infected chickens with S. typhimurium pt. 49.

Chickens sampled	Age (days)	No. of positives	No. of suspects	No. of negatives
Killed cocks	88	2	0	6
,, cock	135	0	0	1
Laying birds at the point of lay	186	2	2	7
Laying birds killed	257	0	0	5
Laying birds killed	307	0	0	6

cocks were negative. At the end of this experiment all birds were negative.

e) Serum immunoglobulins

The results of the immunoglobulin titration determined by ELISA on the sera that were taken at the point of lay are shown in Fig. 21, 22 and 23. The levels of each immunoglobulin from individual chickens against both antigens are shown and compared to those from an SPF pullet of the same age. Birds 3 and 7 were cocks.

Antibody titres varied from bird to bird. In the SPF serum examined IgG levels of 1:150 and 1:600 were present to LPS and surface protein antigens respectively. The level of this immunoglobulin in the infected birds varied from the same level as in the SPF bird to maximum levels of 1:2400 for LPS (hen 6 and 9) and 1:4800 for surface protein antigen (hen 9) (Fig. 21). The highest titre of IgM (1:6400) was obtained in the serum of hen 9 against both antigens (Fig. 23) and in hen 8 against LPS antigen while these levels were 1:800 for LPS and 1:400 for surface protein antigens in the serum of SPF chicken. The lowest levels of IgA (1:200) were found in the sera of the SPF hens and chickens 5 and 7 for both antigens and the highest titre (1:1600) in that of hen 9 for both antigens and hen 6 only for LPS antigen (Fig. 22).

The titres of these immunoglobulins at the end of Experiment 2 are shown in Table 11. Most of the levels had declined since the commencement of lay.

f) Bile and oviduct immunoglobulins

No IgG against LPS and surface protein antigens of S. typhimurium pt. 49 was found in either bile or oviduct fluid from the naturally infected chickens. Using the ELISA test specific IgA and IgM antibodies were both detected in bile and

FIG. 21:

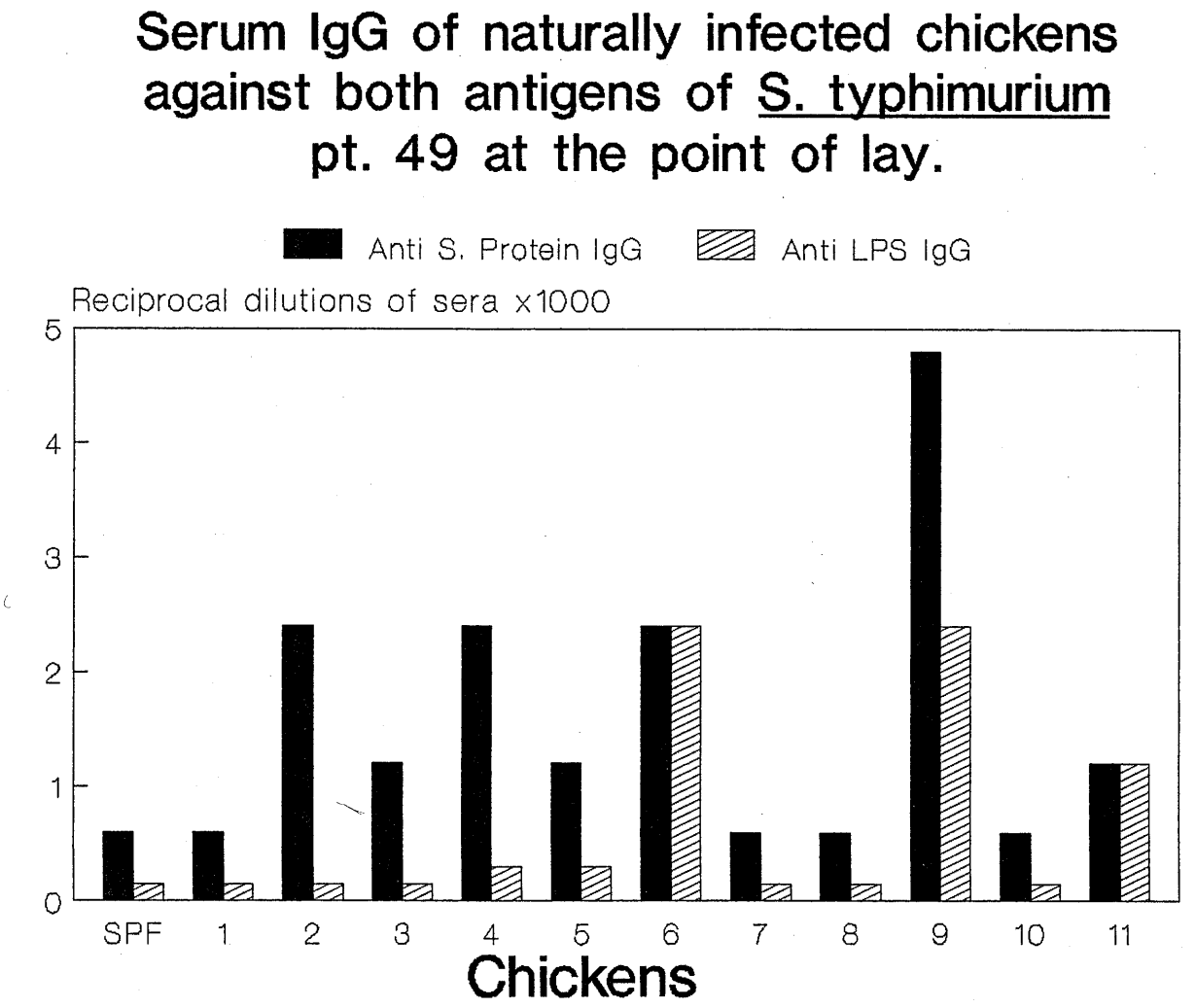

Serum IgG of naturally infected chickens against both antigens of S. typhimurium pt. 49 at the point of lay.
Anti S. Protein IgG
Anti LPS IgG
Reciprocal dilutions of sera x1000
5
4
3
2
1
0
SPF
1
2
3
4
5
6
7
8
9
10
11
Chickens

FIG. 22:

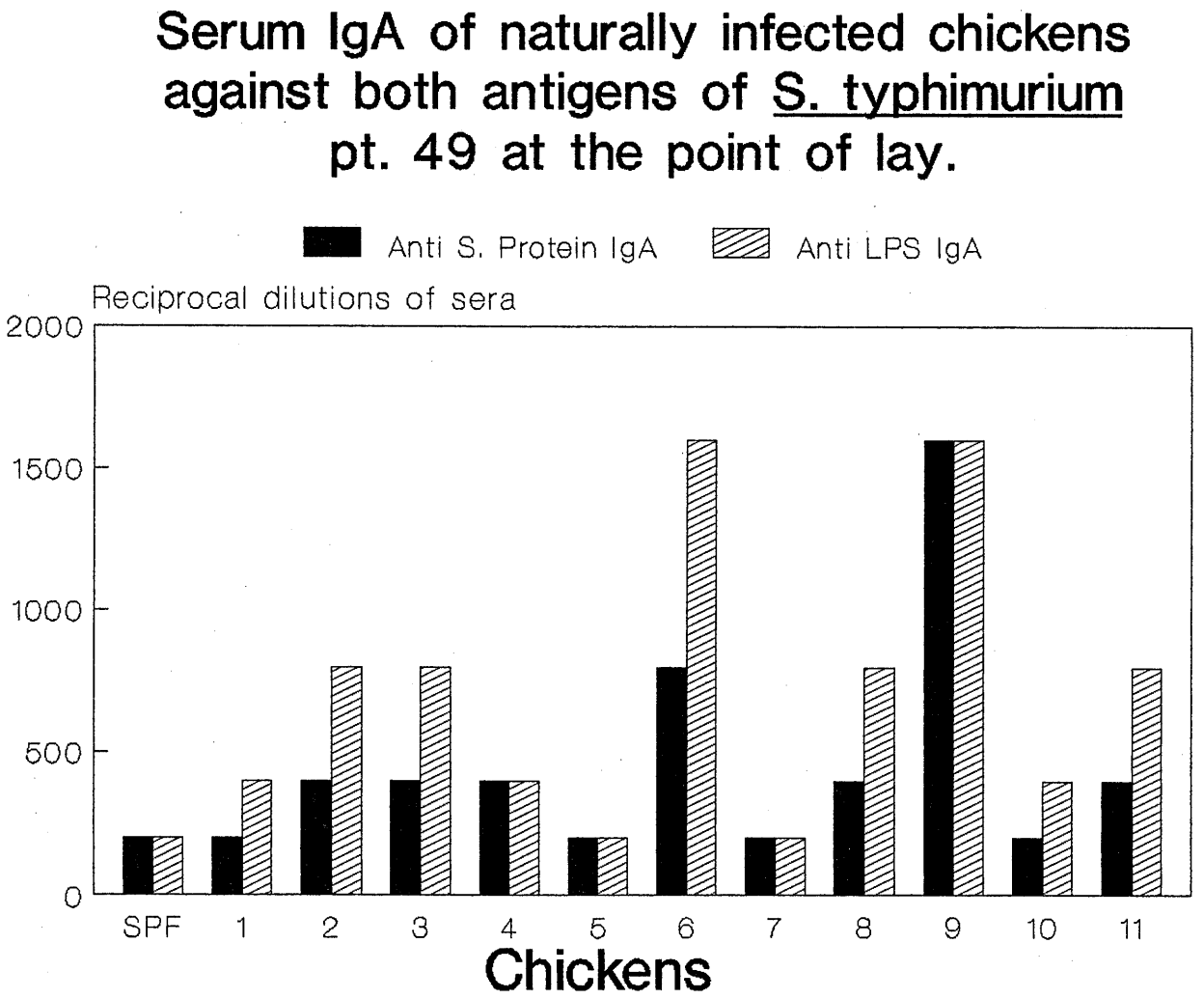

Serum IgA of naturally infected chickens against both antigens of S. typhimurium pt. 49 at the point of lay.
Anti S. Protein IgA
Anti LPS IgA
Reciprocal dilutions of sera
2000
1500
1000
500
0
SPF
1
2
3
4
5
6
7
8
9
10
11
Chickens

FIG. 23:

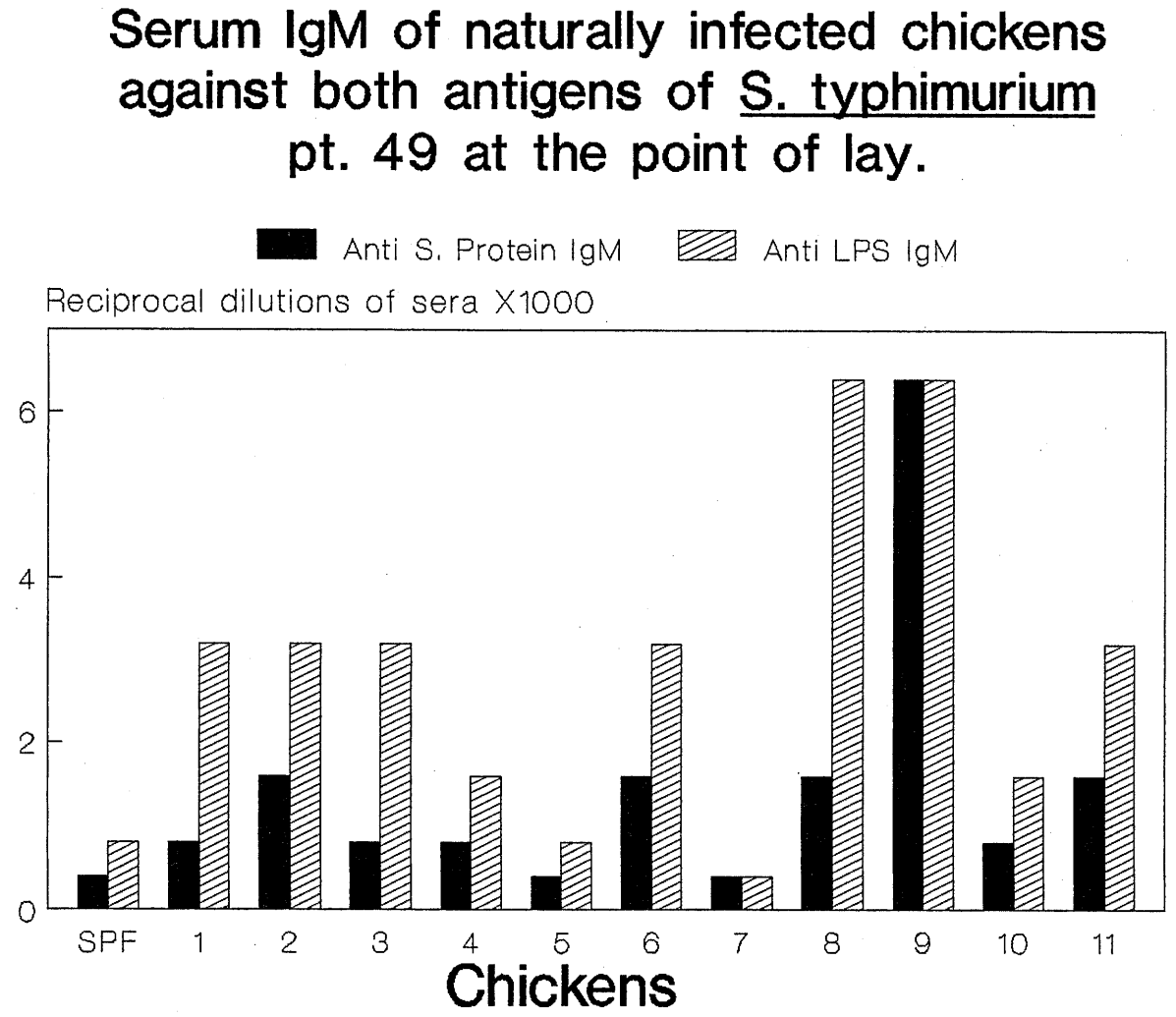

Serum IgM of naturally infected chickens against both antigens of S. typhimurium pt. 49 at the point of lay.
Anti S. Protein IgM
Anti LPS IgM
Reciprocal dilutions of sera X1000
0
2
4
6
SPF
1
2
3
4
5
6
7
8
9
10
11
Chickens

Table 11

Serum immunoglobulins against surface protein and LPS antigens of S. typhimurium pt. 49 from naturally infected chickens on the day of death.

Chicken No.	IgG		IgA		IgM	
	S.P. Ag.*	LPS Ag.	S.P. Ag.	LPS Ag.	S.P. Ag.	LPS Ag.
1	600**	150	200	400	800	1600
2	1200	150	400	800	1600	1600
3	1200	150	400	400	800	800
4	600	75	400	400	400	1600
5	1200	300	200	200	400	800
6	1200	1200	800	800	800	1600
7	600	150	100	200	400	400
8	600	75	400	800	1600	1600
9	4800	2400	800	1600	1600	3200
10	300	75	200	400	400	800
11	1200	1200	400	800	800	1600
SPF	600	150	200	200	400	400

* = Surface protein antigen.

** = Reciprocal dilutions of sera.

oviductal fluid (Tables 12-15). Bile IgA was detected by immunoelectrophoresis but not IgM (Fig. 24)

IgA levels ranged from 1:160 to 1:320 against surface protein antigen and from 1:80 to 1:320 against LPS antigen in bile from the SPF hens (Table 14). The range of bile IgA levels in the infected chickens was 1:1280 to 1:10240 against both antigens and individual variation was observed (Table 12).

The range of bile IgM levels was 1:40 to 1:320 against surface protein antigen and 1:320 to 1:640 against LPS antigen (Table 12). A range of 1:40 to 1:80 was observed for both antigens in the bile of the SPF chickens (Table 14).

The IgA and IgM levels in the oviduct secretions of naturally infected chickens (Table 13) were also higher and wider in range when compared to those of SPF hens (Table 15). The oviduct secretions and bile of hen 9 showed relatively higher immunoglobulin level than others.

g) Post mortem findings

No gross lesions or abnormalities were found to indicate the presence of salmonellosis or any other disease. All hens had a functional ovary and oviduct. 2 hens each had a complete egg in their oviduct and the rest had an incomplete egg.

4 - EGG IMMUNOGLOBULIN AND CHALLENGE STUDIES (EXPERIMENTS 3 AND 4)

A) Materials and methods

a) Eggs

The eggs of naturally infected hens were examined by the ELISA against LPS and surface protein antigens to determine the level of immunoglobulins to S. typhimurium in the white and

Table 12

Bile IgA and IgM antibody to S. typhimurium of naturally infected chickens measured by the ELISA.

Chicken's No.	IgA		IgM	
	Protein Ag.*	LPS Ag.	protein Ag.	LPS Ag.
1	1:1280**	1:1280	N.D.***	N.D.
2	1:2560	1:5120	1:160	1:320
3	1:2560	1:2560	N.D.	N.D.
4	1:1280	1:2560	N.D.	N.D.
5	1:10240	1:10240	1:320	1:640
6	1:5120	1:10240	1:160	1:640
7	1:5120	1:5120	1:80	1:320
8	1:2560	1:2560	N.D.	N.D.
9	1:5120	1:10240	1:320	1:640
10	1:1280	1:2560	N.D.	N.D.
11	1:2560	1:5120	1:160	1:640

* = Surface protein antigen of S. typhimurium pt. 49.

** = The end point dilution as determined by ELISA.

*** = Not done.

Table 13

Levels of IgA and IgM antibody to S. typhimurium in the oviductal secretions of naturally infected hens as determined by ELISA.

Chicken's No.	IgA		IgM	
	Protein Ag.*	LPS Ag.	Protein Ag.	LPS Ag.
1	1:4**	1:16	1:16	1:32
2	1:64	1:256	1:128	1:256
4	1:8	1:8	N.D.***	N.D.
5	1:32	1:256	1:128	1:256
6	1:32	1:256	1:32	1:128
8	1:8	1:32	1:32	1:128
9	1:128	1:512	1:256	1:512

* = Surface protein antigen of S. typhimurium pt. 49.

** = The end point dilution as determined by ELISA.

*** = Not done.

Table 14

Bile IgA and IgM antibody to S, typhimurium measured by the ELISA in the SPF hens.

Chicken's No.	IgA		IgM	
	Protein Ag.*	LPS antigen	Protein Ag.	LPS antigen
1	1:320**	1:320	1:40	1:40
2	1:160	1:160	1:80	1:40
3	1:320	1:320	1:80	1:80
4	1:320	1:320	1:80	1:80
5	1:160	1:320	1:40	1:40
6	1:160	1:80	1:40	1:80

* = Surface protein antigen of S. typhimurium pt. 49.

** = The end point dilution as determined by the ELISA.

Table 15

Titres of IgA and IgM antibody to S. typhimurium determined by the ELISA in the oviduct secretions of SPF hens.

Chicken No.	IgA		IgM	
	Protein Ag.*	LPS antigen	Protein Ag.	LPS antigen
1	1:4**	1:16	1:2	1:8
2	1:16	1:32	1:4	1:8
3	1:8	1:64	1:2	1:32
4	1:16	1:64	1:8	1:64
5	1:16	1:64	1:8	1:64
6	1:8	1:64	1:4	1:32

* = Surface protein antigen of S. typhimurium pt. 49.

** = The end point dilution as determined by the ELISA.

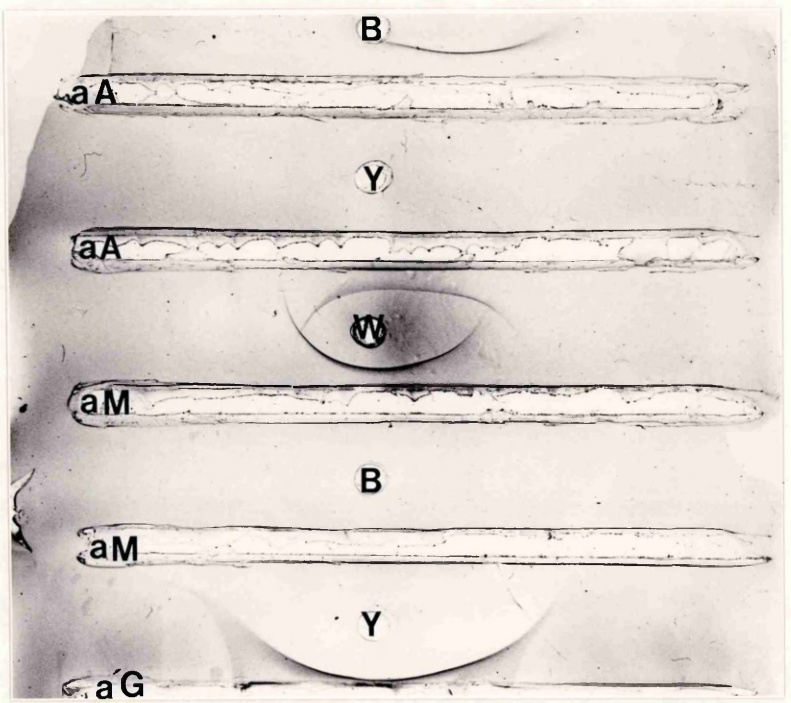


FIG. 24: Immunoelectrophoretic pattern of bile IgA, purified egg white IgA and IgM and purified yolk IgG.

B = Bile W = Egg white Y = Yolk

aG = anti chicken IgG

aM = anti chicken IgM

aA = anti chicken IgA

yolk. These eggs were screened either individually or as a combination of the bulk eggs that were laid in one day. The eggs of those days with the maximum lay were chosen for the study. Due to the lack of some facilities such as trap nests it was not possible to trace the eggs back to the individual hens. Only on day 35th of lay were there 9 eggs and this was not repeated during the period of the experiment. On days 28 and 29 there were 9 eggs together and on days 66 and 67 there were 6and 8 eggs respectively. The egg white and egg yolk of each day was pooled separately and used in bulk in the ELISA studies.

After killing the hens the yolk or the complete egg inside the oviduct was removed carefully. Each egg or yolk was used in the ELISA to determine the level of its immunoglobulin in relation to those in serum and secretions of that individual.

Some of the eggs of these hens were also incubated for challenge studies as described below.

b) Hatching of eggs

A total of 92 eggs from naturally infected hens and 78 SPF eggs were incubated to provide day old chicks for use in challenge studies with S. typhimurium pt. 49. 76 chicks were hatched from 92 eggs of naturally infected hens. 4 chicks were culled due to leg weakness and growth retardation, 3 were killed for serological studies and the remaining 69 were divided into experimental groups as described below. 43 chicks were hatched from 78 SPF eggs. 2 were culled, 3 drowned in the incubator water and 28 were used as control groups in challenge studies. The remaining 10 chicks that hatched at least 24 hours late were killed and their sera were preserved.

c) Challenge studies

Two challenge studies were carried out.

Experiment 3: In this experiment S. typhimurium was given orally to every individual in two groups of 15 (immune group 1) and 8 (immune group 2) chicks that were derived from naturally infected hens. 15 chicks derived from SPF eggs were also dosed with this organism. The inoculation method and instruments used were described in Chapter 2. The inoculum contained $2x10^3$ CFU of S. typhimurium pt. 49 for immune group 1, $0.8x10^3$ CFU for immune group 2 and $0.2x10^3$ CFU for the SPF chicks. These chicks were killed at 4 hourly intervals after inoculation and the distribution of infection in the gut, heart, liver and yolk sac were assessed by direct culture and also after selective enrichment in tetrathionate broth which was subcultured as described in Chapter 2. Caecal contents were used for the determination of Salmonella numbers.

Experiment 4: Two orally inoculated seeder chicks were housed with each group of 13 SPF chicks and 15 chicks from eggs of naturally infected hens. The seeders were dosed with $1x10^3$ S. typhimurium pt. 49 from a fresh nutrient broth culture. A group of 16 chicks derived from immune eggs were also kept as negative controls in another isolated box. The cloacas of all chicks were swabbed daily for a period of 9 days and cultured for Salmonella isolation. Pooled fresh faeces was also used to quantify the shedding of the organism. 5 chicks from each group were killed at 7 day intervals and gut colonisation and systemic infection with the Salmonella was assessed.

B) Results

a) Egg immunoglobulins

Egg transmitted immunity was demonstrated in all 3 immunoglobulin classes and to both LPS and surface protein antigens.

Preliminary studies on the first eggs of this group showed there is variation in immunoglobulin titres among individuals.

On the 35th day of lay 9 eggs probably represented one from each hen. One of the eggs broke and was unsuitable for experimental purposes. The rest of these eggs were screened individually by the ELISA and the results are shown in Figs. 25-27. The titre of each immunoglobulin was different in each egg. The highest titre was for yolk IgG (1:10240) against both antigens. The level of IgA was relatively higher than that of IgM in the white, although the maximum titre against LPS antigen (1:160) was the same for both immunoglobulins. The titre of all 3 immunoglobulins to S. typhimurium was higher than that in SPF eggs except for eggs B, C and F for IgG, and eggs B, F and G for IgA and IgM which contained equal to or lower levels to one or both antigens. There were no IgG antibodies in the egg white and no IgA and IgM antibodies in the egg yolk against both of the antigens. The titres of the immunoglobulins in the eggs that were examined in bulk are shown in Table 16. At all of these points the results were mostly uniform for all 3 immunoglobulin classes and antibody titres higher than those in the SPF control eggs were observed in the infected group.

The IgG levels found in the yolks removed from the oviduct of the 9 hens are shown in Fig 28. Levels were highest in the yolk of hen 9 for both antigens and all except hen 10 showed higher levels than that in SPF control yolk. Table 17 shows the immunoglobulin levels of two complete eggs removed from the oviduct. Hen 5 had higher IgA levels than the SPF control against both antigens but hen 4 only had higher IgM levels against LPS antigen.

b) Challenge studies

The results of Salmonella counts from caecal contents in Experiment 3 are shown in Table 18. The SPF chicks showed the level of 10^8 CFU/g just 4 hours after inoculation which increased to more than 10^9 after 8 hours and remained uniform and constant until the end of the experiment. No Salmonellae

FIG. 25:

Yolk IgG antibody of eggs from infected chickens against both antigens of S. typhimurium pt. 49 on day 35 of laying.

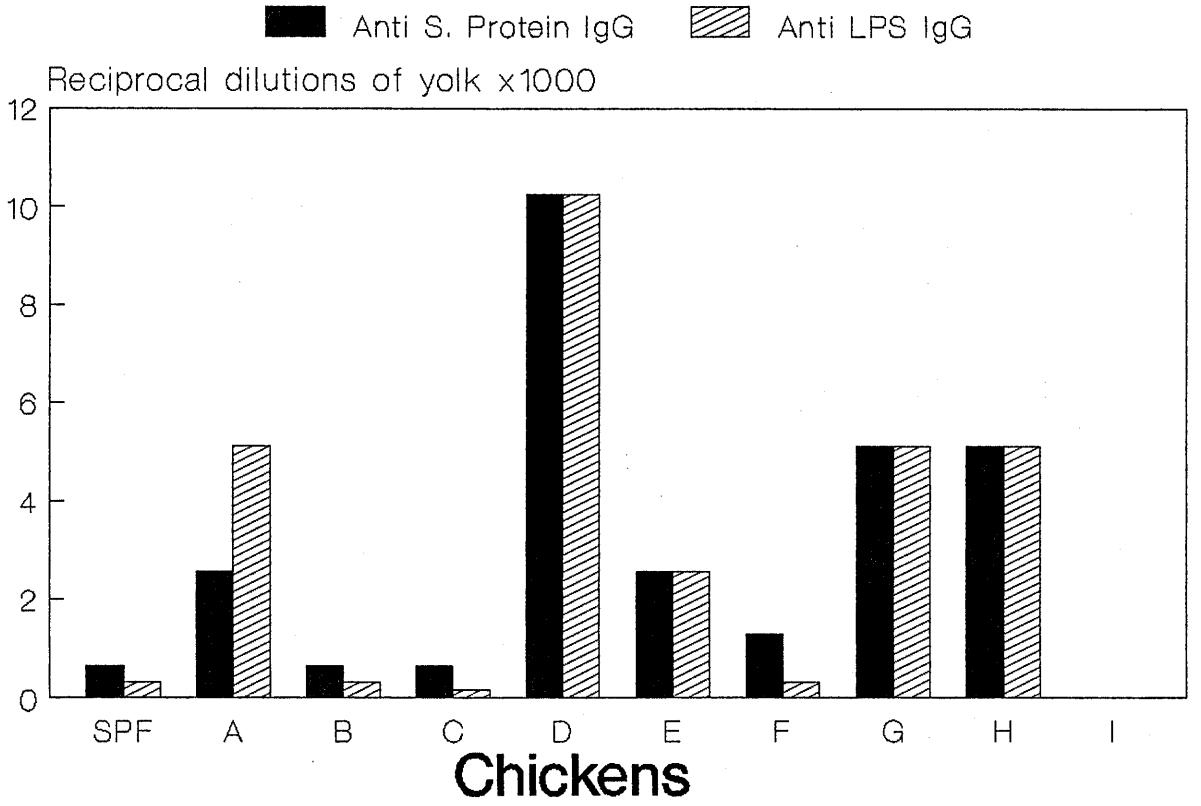


FIG. 26:

Egg white IgA antibody of infected chickens against both antigens of S. typhimurium pt. 49 on day 35 of laying.

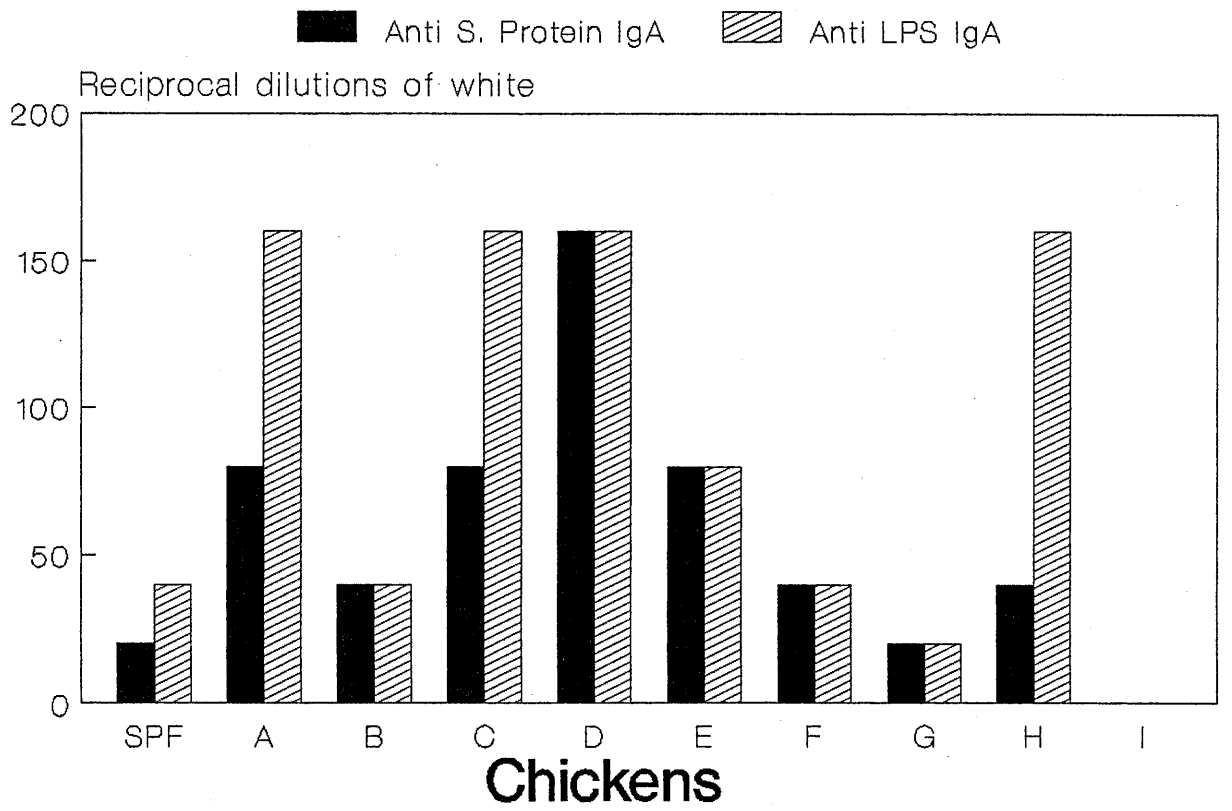


FIG. 27:

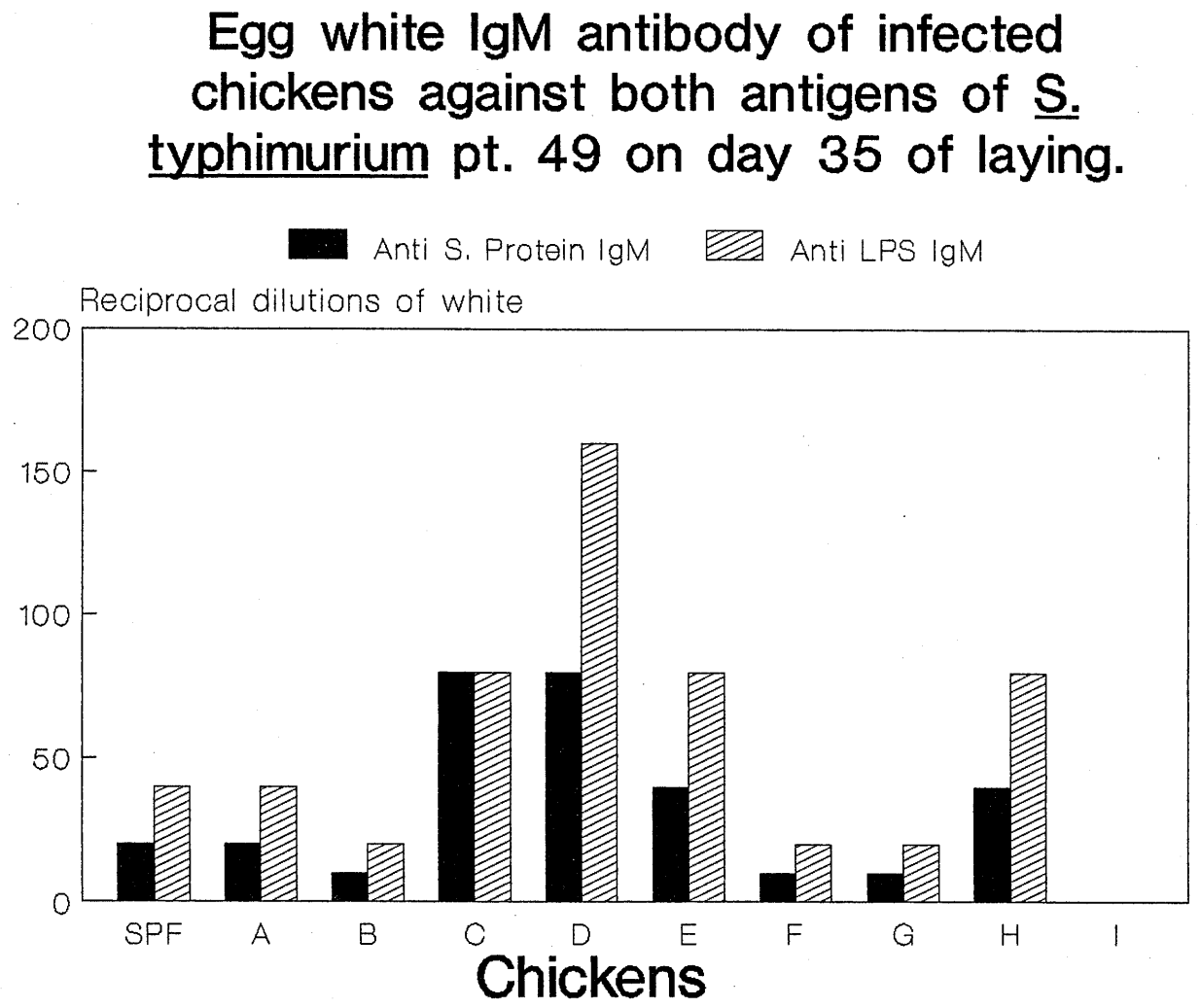

Egg white IgM antibody of infected chickens against both antigens of S. typhimurium pt. 49 on day 35 of laying.
Anti S. Protein IgM
Anti LPS IgM
Reciprocal dilutions of white
200
150
100
50
0
SPF
A
B
C
D
E
F
G
H
I
Chickens

Table 16

Antibody levels to S. typhimurium of the eggs used in the bulk studies.

Day after start of lay	Yolk IgG		White IgA		White IgM	
	S.Pr.Ag.*	LPS Ag.	S.Pr.Ag.	LPS Ag.	S.Pr.Ag.	LPS Ag.
29 (9)**	1:1280	1:2560	1:80	1:160	1:80	1:160
66 (6)	1:2560	1:2560	1:160	1:160	1:160	1:160
67 (8)	1:2560	1:2560	1:160	1:160	1:80	1:160
SPF (5)	1:640	1:320	1:20	1:40	1:20	1:40

* = Surface protein antigen of S. typhimurium pt. 49.

** = Numbers in the parenthesis indicate the number of eggs laid in that day.

FIG. 28:

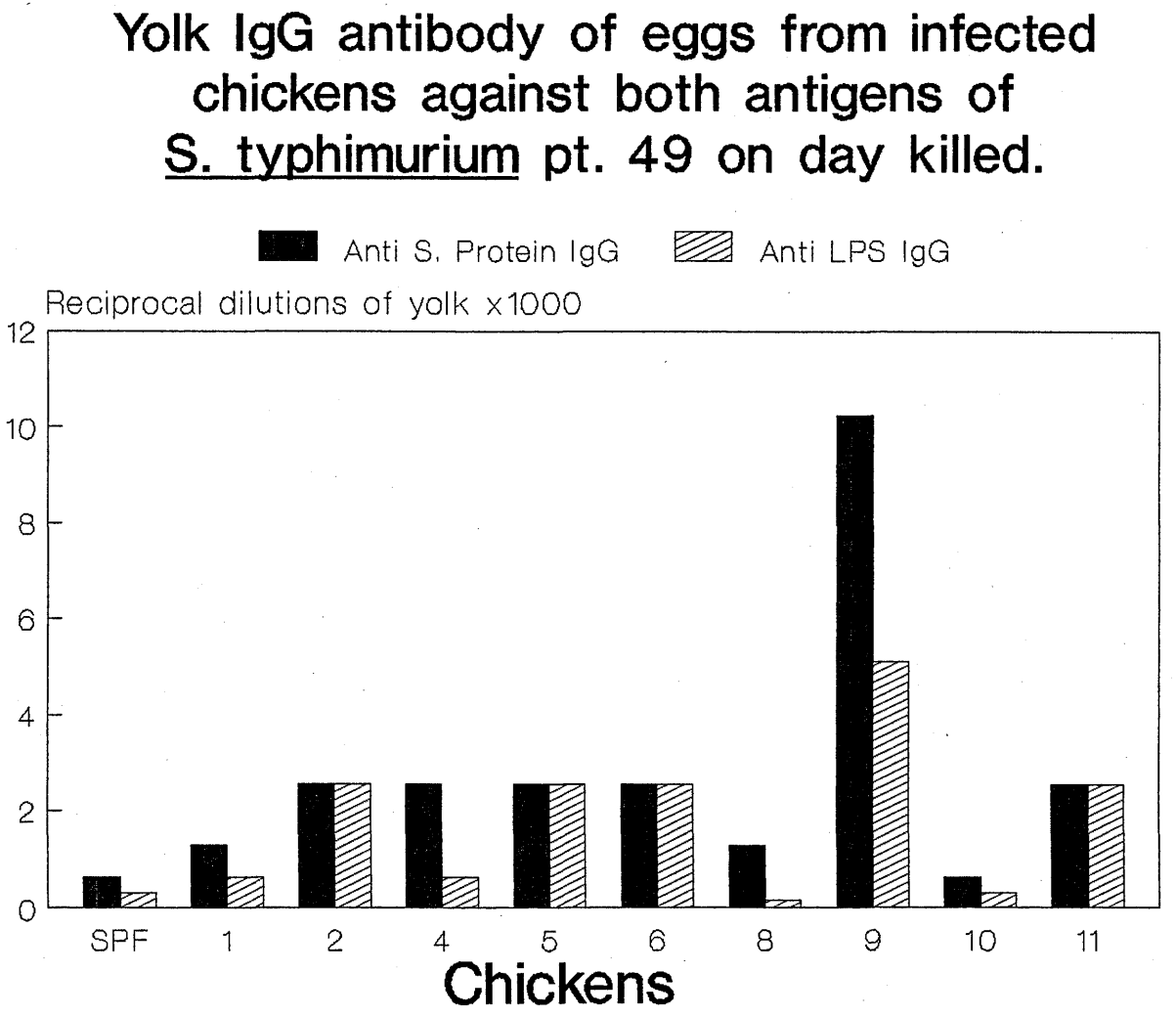

Yolk IgG antibody of eggs from infected chickens against both antigens of S. typhimurium pt. 49 on day killed.
Anti S. Protein IgG
Anti LPS IgG
Reciprocal dilutions of yolk x1000
12
10
8
6
4
2
0
SPF
1
2
4
5
6
8
9
10
11
Chickens

Table 17

Antibody levels to S. typhimurium of complete eggs obtained from the oviduct of naturally infected hens.

Hen No.	Yolk IgG		White IgA		White IgM	
	S.Pr.Ag.*	LPS Ag.	S.Pr.Ag.	LPS Ag.	S.Pr.Ag.	LPS Ag.
4	1:2560	1:640	1:20	1:40	1:20	1:40
5	1:2560	1:2560	1:40	1:160	1:20	1:80
SPF	1:640	1:320	1:20	1:40	1:20	1:40

* = Surface protein antigen of S. typhimurium pt. 49.

Table 18

Salmonella counts from the caecal contents of chicks in Experiment 3.

Hours after infection	Individual chick No.	CFU of Salmonella/g of caecal contents		
		Immune group 1	Immune group 2	SPF
4	1	1×10^{6}	0.00	6.33×10^{8}
	2	0.00	0.00	6.33×10^{8}
	3	0.75×10^{3}	0.00	2.66×10^{8}
	4	-	0.00	-
8	1	4×10^{8}	$< 10^{1}$	1×10^{9}
	2	0.00	$< 10^{1}$	5.33×10^{9}
	3	8×10^{6}	0.00	3.66×10^{9}
	4	-	$< 10^{1}$	-
12	1	1×10^{9}	-	4×10^{9}
	2	4×10^{9}	-	8.66×10^{9}
	3	4×10^{9}	-	6.66×10^{9}
16	1	2×10^{9}	-	4×10^{9}
	2	0.00	-	3.33×10^{9}
	3	6×10^{9}	-	N.D.
20	1	3.5×10^{7}	-	7.66×10^{9}
	2	1.5×10^{9}	-	3.33×10^{9}
	3	2.0×10^{9}	-	N.D.

N.D. = Not done.

CFU = Colony forming units.

were isolated from the immune group 2 (dosed with $0.8x10^3$) at 4 hours and only 0.0 to <10 CFU/g were isolated after 8 hours. Immune group 1 (dosed with $2x10^3$) started with the level of 0.0 to 10^6 at 4 hours which increased to 10^8 after 8 hours and reached more than 10^9 at 12 hours after inoculation. The quantity of Salmonella isolated was not uniform among the individuals at any given time.

The results of Salmonella isolation from different organs are shown in Table 19. No Salmonella were isolated from the heart and liver of immune chicks up to the end (20 hours) of Experiment 3, while the SPF chicks showed infection of the liver and heart after 20 hours post-inoculation. The caeca were the only organs colonised by the Salmonella 4 hours after inoculation and were heavily populated with the organism. Colonisation of the caeca was delayed in chicks of both immune groups when compared with the SPF controls (Table 18 and Fig. 29). The difference had disappeared by 12 hours post infection in most cases but uncolonised birds were still present (chick 2, 16 hours, immune group 1, Table 18).

The results of Experiment 4 are shown in Tables 20-21 and Figs. 30-31. Uninfected controls (negative controls) remained free from infection. Cloacal swabbing indicated that faecal shedding was delayed in the chicks from immune eggs (Figs. 30-31). Systemic infection could not be demonstrated in the chicks from the infected hens but was present in those from SPF hens. One of the immune chicks (No. 842) that did not show infection by cloacal swabbing was included in the group that were killed on day 7 post exposure. No Salmonella could be isolated from its heart, liver, jejunum, ileum and caecum.

Attempts to count the Salmonellae in the caecal contents of these chicks on day 21 showed that 2 out of 3 chicks from SPF hens had 10^7 CFU/g while there was no Salmonella growth up to the level of 10^{-6} dilution of caecal contents from the chicks derived from infected hens. In the lowest dilution there

Table 19

Salmonella isolation from the organs of the chicks killed at different time intervals in Experiment 3.

Hours after infection	Organs cultured (1)	Immune group 1		Immune group 2		SPF	
		Direct (2)	Indir. (3)	Direct	Indir.	Direct	Indir.
4	Heart	0:3*	0:3	0:4	0:4	0:3	0:3
	Liver	0:3	0:3	0:4	0:4	0:3	0:3
	Yolk sac	0:3	0:3	0:4	0:4	0:3	0:3
	Jejunum	0:3	0:3	0:4	0:4	0:3	0:3
	Ileum	0:3	0:3	0:4	0:4	1:3	1:3
	Caecum	2:3	2:3	0:4	0:4	3:3	3:3
8	Heart	0:3	0:3	0:4	0:4	0:3	0:3
	Liver	0:3	0:3	0:4	0:4	0:3	0:3
	Yolk sac	0:3	0:3	0:4	0:4	0:3	0:3
	Jejunum	1:3	1:3	0:4	0:4	2:3	2:3
	Ileum	0:3	0:3	0:4	0:4	1:3	1:3
	Caecum	2:3	2:3	3:4**	3:4	3:3	3:3
12	Heart	0:3	0:3			0:3	0:3
	Liver	0:3	0:3			0:3	0:3
	Yolk sac	0:3	0:3			0:3	0:3
	Jejunum	0:3	0:3			1:3	1:3
	Ileum	1:3	1:3			1:3	1:3
	Caecum	3:3	3:3			3:3	3:3
16	Heart	0:3	0:3			0:3	0:3
	Liver	0:3	0:3			0:3	0:3
	Yolk sac	0:3	1:3			2:3	2:3
	Jejunum	0:3	0:3			3:3	3:3
	Ileum	0:3	0:3			3:3	3:3
	Caecum	2:3	2:3			3:3	3:3

Table 19 continued:

	Heart	0:3	0:3			1:3	2:3
	Liver	0:3	0:3			2:3	1:3
20	Yolk sac	1:3	1:3			1:3	1:3
	Jejunum	1:3	1:3			2:3	3:3
	Ileum	1:3	1:3			3:3	3:3
	Caecum	3:3	3:3			3:3	3:3

(1) = Salmonella was the only organism isolated from heart, liver and yolk sac as shown.

(2) = The samples were directly cultured on sheep blood, MacConkey and BG agar without enrichment.

(3) = The samples were cultured into tetrathionate broth and then subcultured onto DCA, SS and BG agar.

* = No. of positives:Total No. cultured.

** = Few colonies were grown on the BG agar only.

FIG. 29:

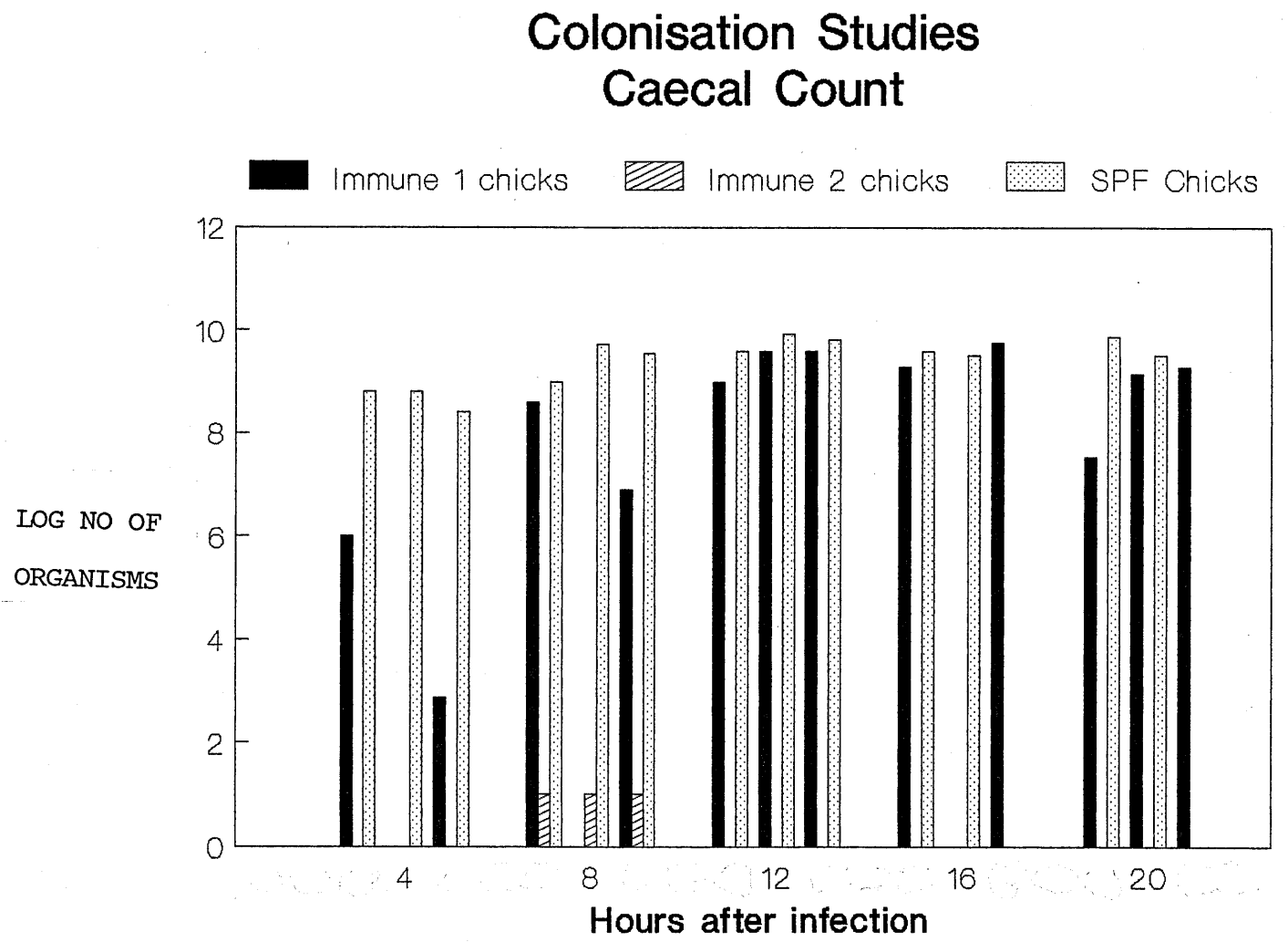

Colonisation Studies
Caecal Count
Immune 1 chicks
Immune 2 chicks
SPF Chicks
LOG NO OF ORGANISMS
0
2
4
6
8
10
12
4
8
12
16
20
Hours after infection

Table 20

Results of cloacal swab culture for Salmonella isolation from chicks in Experiment 4.

Days after exposure	Immune group		Control group	
	No. positives :Total birds	% Infected	No. positives :Total birds	% Infected
0	0:15	0.00	0:13	0.00
1	2:15	13.33	4:13	30.76
2	3:15	20.00	7:13	53.85
3	5:15	33.33	11:13	84.61
4	8:15	53.33	13:13	100
5	10:15	66.67	13:13	100
6	11:15	73.33	N.D.	---
7	N.D.	---	N.D.	---
8	N.D.	---	8:8	100
9	10:10	100	N.D.	---

N.D. = Not done.

Table 21

Salmonella isolation from the organs of the chicks killed at different times in Experiment 4.

Days after infection	Organs cultured (1)	SPF group		Immune group		Control group	
		Direct (2)	Indir. (3)	Direct	Indir.	Direct	Indir.
7	Heart	0:5*	0:5	0:5	0:5	0:5	0:5
	Liver	1:5	2:5	0:5	0:5	0:5	0:5
	Yolk sac	N.D.	0:1	0:2	0:2	N.P.**	N.P.
	Crop	0:5	3:5	0:5	2:5	0:5	0:5
	Jejunum	0:5	0:5	0:5	0:5	0:5	0:5
	Ileum	0:5	2:5	1:5	1:5	0:5	0:5
	Caecum	0:5	5:5	0:5	4:5	0:5	0:5
14	Heart	1:5	1:5	0:5	0:5	0:5	0:5
	Liver	0:5	1:5	0:5	0:5	0:5	0:5
	Crop	0:5	2:5	0:5	3:5	0:5	0:5
	Jejunum	0:5	1:5	0:5	0:5	0:5	0:5
	Ileum	0:5	1:5	0:5	2:5	0:5	0:5
	Caecum	2:5	5:5	0:5	5:5	0:5	0:5
21	Heart	0:3	0:3	0:5	0:5	0:6	0:6
	Liver	0:3	0:3	0:5	0:5	0:6	0:6
	Crop	0:3	1:3	0:5	2:5	0:6	0:6
	Jejunum	0:3	0:3	0:5	0:5	0:6	0:6
	Ileum	0:3	0:3	0:5	1:5	0:6	0:6
	Caecum	0:3	2:3	0:5	5:5	0:6	0:6

(1) = Salmonella was the only organism isolated from heart, liver and yolk sac as shown. (2) = The samples were directly cultured on sheep blood, MacConkey and BG agar without enrichment. (3) = The samples were cultured into tetrathionate broth and then subcultured onto DCA, SS and BG agar.

* = No. of positives:Total No. cultured. ** = Not present.

FIG. 30:

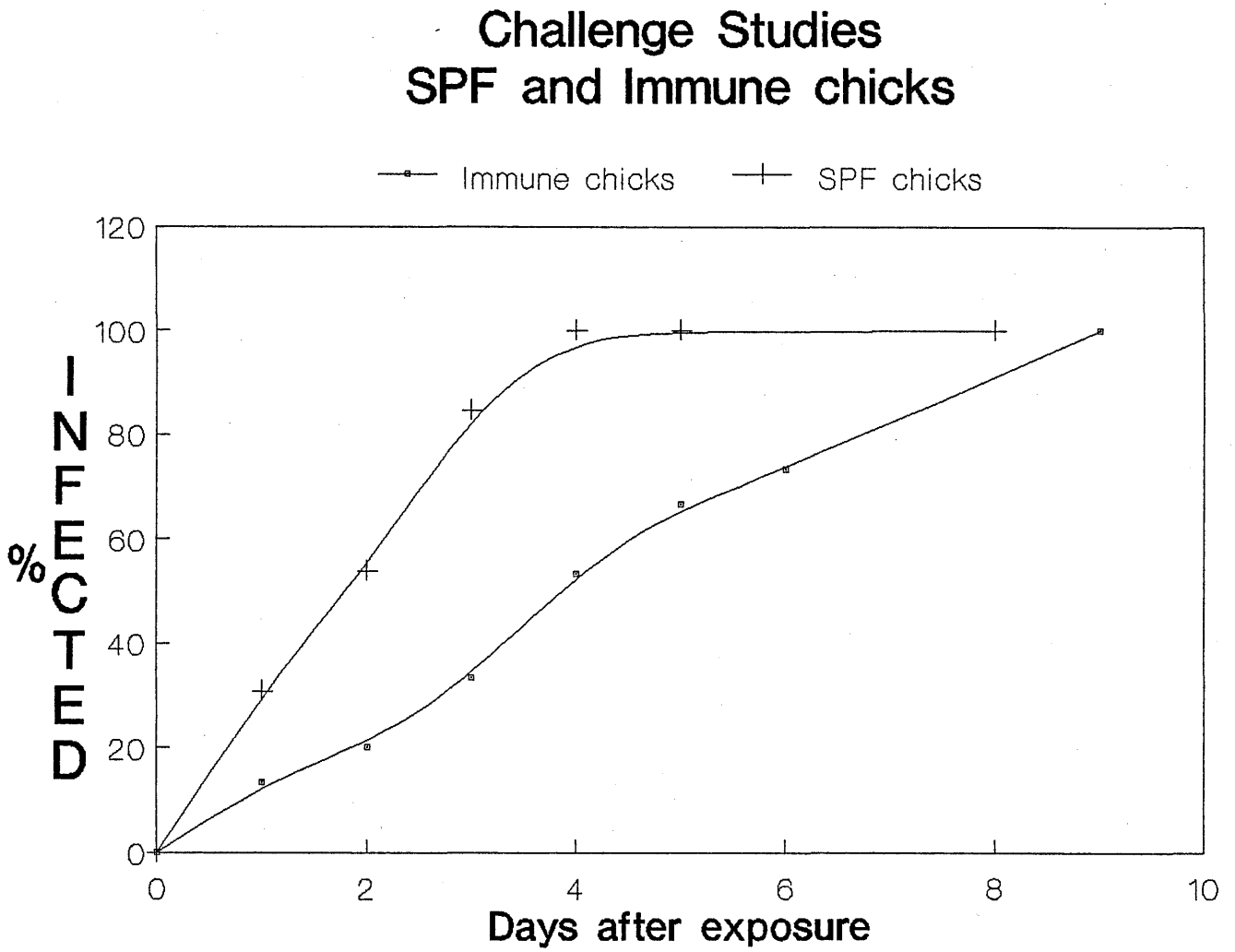

Challenge Studies
SPF and Immune chicks
Immune chicks
SPF chicks
% INFECTED
120
100
80
60
40
20
0
0
2
4
6
8
10
Days after exposure

FIG. 31: Photograph of an overnight culture of a cloacal swab from chick 842 from the immune group (A) and chick 342 from the control group (B) directly onto BG agar. Swabs were taken on day 5 post exposure. Only Salmonella colonies are present on B while only coliforms were grown on A.

was over-growth of other bacteria mostly Enterobacter cloacae.

No Salmonellae could be isolated from any of the unhatched eggs, dead embryos, culled chicks or cloacal swabs of the experimental chicks prior to infection.

5 - DISCUSSION

The objectives of this study were:

a) To determine the capability of the strain of S. typhimurium pt. 49 used to colonise the gut and to produce systemic infection.

b) To examine sera, eggs, bile and oviductal washing of the infected chickens for specific antibodies against antigens of this organism.

c) To evaluate the role of natural immunity in the protection of young chicks from infection with same organism.

A) Colonisation studies

Studies showed that S. typhimurium pt. 49 was capable of colonising the chick intestine. An inoculum of $1x10^3$ CFU of the organism resulted in infection with 9.5% mortality in the initial colonisation experiment (Experiment 1) and later this was repeated with $0.2x10^3$ CFU in Experiment 3. The speed of infection in contact birds was surprising (less than 24 hours in the uninfected group of birds housed in the same pen). 100% gut colonisation was observed either in orally inoculated birds or in the contact birds. 76.19% of the orally inoculated birds in Experiment 1 were systemically infected (Table 4). The level of Salmonella shedding just after infection was more than 10^9 CFU/g of fresh droppings in both inoculated and contact birds (Table 3) and continued at this level until at least day 11. The level of shedding declined thereafter but the organism was

isolated from the faeces until 135 day of age (Table 8) but was detectable by cloacal swabbing at 186 days of age (Table 7). The organism was isolated from crop, gizzard, proventriculus, jejunum, ileum, caecum and and rectum but not the duodenum of colonised birds (Table 5) and the highest levels were obtained from the contents of the rectum and caecum (Table 5).

Systemic infection was demonstrated in both groups (Tables 4, 5 and 10). Salmonella was isolated from heart, liver, spleen but not from bile (Tables 5 and 9). The liver yielded Salmonellae most frequently and in greatest numbers and was chosen for Salmonella isolation in the rest of the study. Similar findings were obtained for the spleen, but the small size of this organ made it less suitable for monitoring purposes. The intestine became colonised in less than 4 hours in the day old SPF chicks of Experiment 3 (Table 18). This finding meant that later studies of colonisation had to be carried out at short intervals and could, therefore, only use small numbers of birds. Systemic infection was observed at 20 hours after inoculation of the organism into the crop of the chick (Table 19).

Chicks that were colonised and shedding only Salmonella organisms were used in the E.M. studies and light microscopical studies. Bacteria with the morphology of Salmonellae were seen in the gut but direct attachment to epithelial cells was not seen in the caecum in electron micrographs (Fig. 16). Organisms were closely associated with the brush borders in the small intestine (Fig. 10). The high level of Salmonella in the faeces and caecal contents (Tables 3 and 5), isolation of the organism from most parts of the gut (Table 5) and shedding for a long period in the faeces proved that the strain of Salmonella used could colonise the gut. In this it resembles other strains of S. typhimurium (Snoeyenbos et al. 1982, Barrow et al. 1987a). As this organism was isolated from a colonised broiler flock it can be concluded that passage on synthetic media and lyophilization did not alter the virulence of the bacteria for

young chicks.

The results indicate that objective"a" was successful and repeatable. The model was therefore adopted for the remaining studies.

B) Antibody in hens

a) The chicken

As explained above, the control groups in the Experiment 1 acquired the infection unintentionally and their infection was regarded as natural infection. These naturally infected birds were reared to adulthood for egg production. They carried the organism for a long time (6 months) and were still carrier after the commencement of lay. This part of the experiment was repeated successfully and in the second trial, 5 out of 11 chickens were shedding the organism at the point of lay. The chance of multiple infection was reduced by keeping the birds in isolation and providing irradiated feed. No other pathogen was isolated from the faeces of these birds in aerobic cultures. No clinical signs of any disease were observed during the experiment and no gross pathological lesions were found at the time of slaughter to indicate the presence of other diseases.

b) The ELISA

The ELISA technique was used to determine the presence of specific immunoglobulins against antigens of S. typhimurium pt. 49. The ELISA adopted in this study was a heterogenous, indirect, double antibody ELISA as described in Chapter 2. The reasons for using this technique were its simplicity to perform, ease of application to different samples, high sensitivity and specificity and reasonable cost. The results obtained were also reliable and repeatable as described below.

(i) Solid phase

The polystyrene microplates (Immulon A and B) used have been recommended and used by most investigators (Prusak-Sochaczewski and Loung 1989, Nieto et al. 1986, Nardiello et al. 1985, Burrells and Dawson 1982, Donachie and Jones 1982, Voller et al. 1976). Flat bottom plates were chosen so that they could be read accurately in the spectrophotometer (Burrells and Dawson 1982).

Coating of both type of plates was carried out at pH 9.6 (carbonate-bicarbonate buffer) and 4^{o}C for a period of 18-24 hours (for both antigens). Under these conditions a maximum saturation value was obtained which was not affected by increasing the concentration of antigen or the duration of incubation up to 72 hours. The required concentration (optimum concentration to give an absorbance reading above 1.0) was found. The sensitivity of the test using different concentrations of each antigen is shown in Figs. I-IV (Appendix 1).

No protein contamination of the LPS antigen could be demonstrated by SDS PAGE (Fig. 6). M129 B (Immulon B) plates are a general purpose plate to which both protein and LPS antigens could be adsorbed. The purity of the LPS used was therefore a key factor in the prevention of non specific binding at coating time. M129 A (Immulon A) plates are designed for adsorbtion of protein antigens and are unsuitable for adsorbtion of LPS. Any contamination of the surface protein antigen with LPS antigen (Fig. 7) was considered to be eliminated by this means. This supposition was confirmed by the test described in Chapter 2, Section 2. Therefore the effect of any contamination of the surface protein antigen with LPS was overcome by using M129 A plates.

(ii) Antisera

Although there is cross reaction between the light chains of chicken IgA, IgM and IgG, no such reaction has been reported between heavy chains. The antisera used in this study were all Fc specific and were raised in goats against the Fc part of the chicken immunoglobulins. The optimum working dilution for each one was found as described in Chapter 2 and this dilution was used in all experiments. All the antisera used were from the same source and the same batch. New vials were examined for the optimum working dilution prior to use. No non specific binding was observed in control wells with no sample. At least 2 such control wells were used on every plate for each antiserum during the study. Figs. V-X (Appendix 1) show the correlation between absorbance and different dilutions of positive and negative standard sera with the optimum working dilution of each antiserum.

(iii) Conjugate

The main parameter in the performance of the enzymes in an ELISA is their specific activity. In this study horse radish peroxidase conjugated rabbit anti goat IgG (H+L) was purchased commercially. It was examined for its specificity in the ELISA system used in this study. At the time (30 minutes) when the readings for positive samples were highest, those for control wells (The wells with no sample and no antisera and the wells with no antisera only) were zero. Duplicate control wells were used on each plate as described above. The high dilution (1:10,000) at which the conjugate worked indicates the sensitivity of the enzyme and test.

(iv) Diluting and washing buffers

Washing buffer was used to remove the unadsorbed antigens and unabsorbed immunoglobulins and conjugates from the wells to prevent their interference with subsequent assay procedures.

Tween 20 was included in both buffers as a blocking agent for the prevention of non specific binding. However, as this detergent alone may cause desorption of proteins (Prusak-Sochaczewski 1989), low concentrations (0.05% v/v) were used in both buffers and instead, bovine serum albumin (0.25% w/v) was included in the diluting buffer. In practice these 2 were not 100% effective and finally skimmed milk powder (5% w/v) was also included to increase the specificity of the test.

c) The immunoglobulins

(i) Serum

Specific IgA, IgM and IgG to both antigens of S. typhimurium pt. 49 were found in the serum of infected chickens. The ELISA results show that the birds had variable levels of antibody. These levels were as low as those in the SPF serum and as high as 1:4800 for IgG (Fig. 21, hen 9), 1:1600 for IgA (Fig. 22, hens 6 and 9) and 1:6400 for IgM (Fig. 23, hens 8 and 9). This finding is of considerable interest because it shows that serum antibody could be used to detect infected flocks but not, perhaps, individual infected birds. The reason for the variation in levels is not clear at this time. It may reflect the initial systemic infection of the birds. Those with highest antibody levels may have had persisting systemic infection or have developed such infections later in life (chicken 9). High levels of IgM (hens 8 and 9) could be a sign of recent systemic infection. The low levels found may come from birds which only became colonised in their intestine. The highest titre (1:6400) was of IgM antibody to both surface protein and LPS antigens in hen 9. Levels of IgG were lower and those of IgA lower still (1:1600).

The results suggest that IgG or IgM detection would be of most value in detecting the presence of immune chickens in a flock. The presence of specific IgG and IgM to S. typhimurium, S. enteritidis, S. anatum and S. infantis in the sera of BALB/c

mice was reported by Kudrna et al. (1987). These mice were already vaccinated with outer membrane protein and LPS of these organisms.

(ii) Eggs

Variations were observed in the levels of the specific immunoglobulins present in the eggs of infected hens as in their sera. The high levels of specific IgG in some egg yolks indicates direct transfer of this immunoglobulin from serum to the yolk as reported by Kowalczyk at al. (1985), Rose et al. (1974) and Kramer and Cho (1970). Due to the lack of trap nests it was not possible to identify the eggs of individual hens and the results obtained from the individual eggs cannot be traced back to individuals.

The yolks obtained from the oviducts of the hens at the time of slaughter show a correlation between their IgG level (Fig. 28) and that in the serum (Table 11). The level of IgG in the yolk was higher than that in the serum of the same hen against both antigens. Specific IgA and IgM were only present in the white. 4 eggs had higher levels of these immunoglobulins than SPF eggs on day 35 of laying against both antigens. This suggests that infection with Salmonella can stimulate local antibody production in the oviduct. Due to lack of facilities and absence of complete eggs from the oviducts of the hens at slaughter, the correlation between these levels and those of the hen serum (if any) or oviduct secretions cannot be determined.

(iii) Oviduct

Only IgA and IgM specific antibodies were found in the oviductal secretions as in the egg white. The hens with higher levels of IgA and IgM in their sera (Table 11) had higher levels of these immunoglobulins in the oviduct secretions too

(Table 13). It is considered unlikely that these originate from the serum (Dohms 1978) and they were probably produced by the local IgA and IgM producing cells in the oviduct described by Lebacq-Verheyden et al. (1972b). No IgG was found in the oviduct secretions.

(iv) Bile

The presence of IgA in the bile is reported by all investigators, but the presence of IgM is in controversy. Using immunodiffusion and immunoelectrophoresis, Sanders and Case (1977) showed that IgA is the only immunoglobulin of the bile. Mockett (1986) reported the isolation of IgM from bile by affinity chromatography and PAGE. The difference could be due to the sensitivity of the technique used. As shown in Fig. 24, immunoelectrophoresis did not show IgM in the bile, but the ELISA revealed high levels of specific IgM in the bile against both antigens of S. typhimurium. The presence of specific IgM to S. enteritidis in bile is reported by Jackson and Walker (1983) in rats that were inoculated with killed organism. The absence of IgG suggested that the IgM demonstrated here was not the results of contamination with serum at sampling.

C) Challenge study with natural immunity

Small groups of chicks were used to study the role of natural immunity in resistance to infection with S. typhimurium pt. 49. The reasons for the small size of the groups used were a) a low number of laying hens and the decreased hatchability of the eggs after 7-10 days storage b) the difficulty of sampling a large number of chicks (especially monitoring at 4 hourly intervals). The figures presented are not analysed statistically and are mainly evaluated qualitatively. Seeders were used in Experiment 4 in order to produce conditions similar to those in poultry houses, where the infection spreads through the flock by means of infected birds. This is also the natural route of transfer of the

organism to exposed chicks.

The results of challenge studies confirmed that natural antibody transferred from hens to eggs could provide immunity to young chicks. In both challenge experiments no systemic infection was demonstrated in the chicks from immune eggs. In Experiment 3 systemic infection was only found in the SPF controls at the end of study (20 hours). In Experiment 4 which lasted longer (21 days) the protection by maternal immunity was more obvious. The effects of immunity on colonisation were less clear cut. The result of Experiment 3 (Table 18) suggested that colonisation of the intestine was delayed or even prevented. The variability in results (immune group 1, Experiment 3) may have resulted from the variation in the levels of antibodies found in the eggs (Figs. 25-28). At this stage it is not possible to predict the role of different immunoglobulin classes in this protection. In Experiment 4 this delay was shown to be present even at day 1 and to disappear between days 6 and 9. These findings suggest that faecal shedding of Salmonella can be dramatically reduced by the presence of antibody in the eggs.

The phenomenon demonstrated here suggests that egg transmitted immunity is a potential method of protecting the newly hatched chicks against both systemic infection and intestinal colonisation during the vulnerable hatching period. This confirms the original suggestions provided for turkey poults by Thain et al. (1984) using S. hadar.

CHAPTER 4

VACCINATION STUDIES IN THE PROTECTION OF THE CHICK AGAINST INFECTION AND COLONISATION WITH SALMONELLA TYPHIMURIUM PHAGE TYPE 49.

1 - INTRODUCTION

The results of the studies in Chapter 3 indicated that natural infection with S. typhimurium pt. 49 in the chicken can increase the level of specific immunoglobulins in the sera, secretions and eggs of some of the infected birds. Chicks derived from eggs containing these immunoglobulins were protected against infection with the same organism. The literature review in Chapter 1 indicated that the role of humoral immunity in protection against Salmonella infection was not clear and the effect of vaccination of the dam with this organism for the purpose of immune chick production had not yet been studied.

This Chapter describes the production of two killed Salmonella vaccines and their role in stimulating of antibody production, the transfer of specific antibodies into the egg and the part these play in the protection of day old chicks against infection with the same organism.

2 - VACCINATION STUDY (EXPERIMENT 5)

A) Materials and methods

a) Chickens

(i) History

Ten pullets and two slightly older cockerels were purchased from Wickham SPF farms for the vaccination study. These chickens had been hatched and reared as SPF at that farm

up to 16 weeks of age and were then sent to Glasgow by Red Star Parcel train. Data from the 6 SPF hens described in Chapter 3 was used as the control in this study. The flock(s) of origin had been monitored for most poultry viral and mycoplasmal diseases at regular intervals according to the certificate supplied with the birds (Appendix 3). Testing for salmonellosis was as follows:

The serum plate agglutination test was used monthly for the detection of S. pullorum infection. 1 composite faecal sample per 2 cages (maximum 10 samples in any month) was examined by the selective enrichment method to screen the flock for other Salmonella species. These tests were all negative for the flock(s) of origin of the chicken supplied.

The feed used at Wickham had been disinfected by methyl bromide but no information about its content of Salmonella antigen was available. The birds had been maintained on a continuous 15 hour light programme. Before purchase their beaks had been clipped.

(ii) Accommodation during the experiment

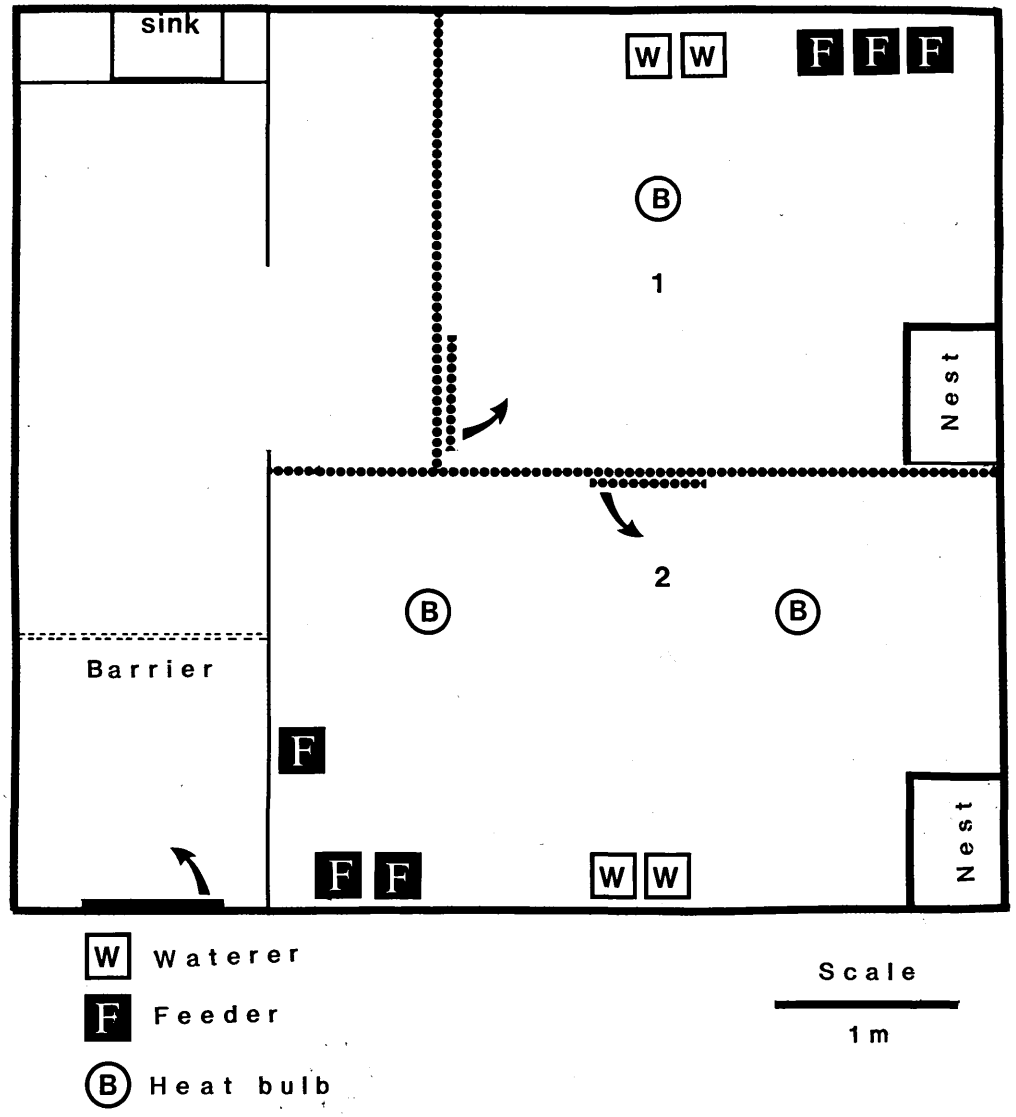
An isolation unit 4.9m wide, 5.4m long and 2m high was divided into 2 parts (Fig. 32) using wire mesh. The height of this partition was 2m and it was solid up to 0.5m from the floor. 3 infra-red light bulbs were hung 1.5m above the floor to produce heat and light. An extra wall heater was mounted on the wall inside the entrance hall 2.5m above the floor. Wood shavings were used as litter. Feeders, waterers and nests were placed in both pens as shown in Fig. 32.

(iii) Sanitation and Hygiene

During the period of experiment the birds were attended by people with no contact with infected birds. Clean overalls and boots were available at the entrance hall before the barrier

FIG. 32:

Plan of chicken accommodation for vaccine study



(Fig. 33) and a disinfectant dip was placed just after the barrier. Access was possible only after passing through the disinfectant. The disinfectant used was Carbo disinfectant soap (Union Chemical Company Ltd., Carronshore, Falkirk, Stirlingshire) at a concentration of 1 fluid oz. per gallon of warm water. Cold and hot (60^{o}C) water and soap was available at the sink (Fig. 32).

Litter, feeders, waterers, nests and all other equipment had been cleaned and were placed inside the unit and fumigated with 0.3% Tegodor 48 hours before the arrival of chickens. Wood shavings were sterilised with formaldehyde and gluteraldehyde gas and then taken into the unit in clean, sterile plastic bags. Any equipment that was required to be taken into the unit was sterilised first.

Boxes of feed were stored inside the entrance hall, in front of the barrier. The plastic bag holding the feed was removed carefully and hygienically and taken inside the unit at the space adjacent to partition No. 1. Samples were taken just after opening the bag as described in Chapter 2.

All the doors and windows to the unit were sealed off to prevent the entrance of insects, small birds and rodents. Supakill insecticide (B.P. Sanmex Co. Ltd., Glasgow) was used to keep the unit free from insects. Disposable gloves and masks were used inside the unit. The chickens faeces and litter were monitored for Salmonella as described below. After arrival the chickens were divided into 2 groups of 5 hens and one cock and each group was accommodated in one pen (Fig. 34) Each chicken was individually identified by a wing tag number placed in the wing web.

(iv) Feed and water

Feed and water were freshly supplied every day and were available ad-lib The feed was diet breeder pellets supplied by

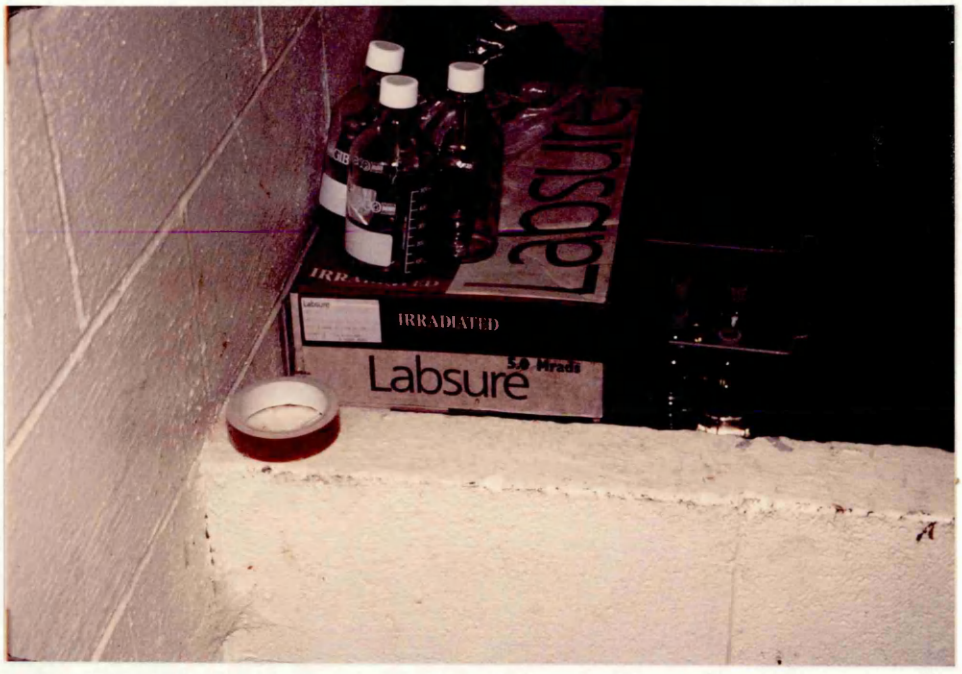


FIG. 33: Photograph of the barrier in the entrance hall to the pen shown diagrammatically in Fig. 32. Feed and bottles of boiled water were stored on the outside of the barrier.

FIG. 34: Photograph of the chickens in group 1 (parenterally-orally vaccinated). Two feeders and a nest box are shown.

Labsure and was gamma irradiated at 5 M Rads (Appendix 2). For the first 3 days of the experiment autoclaved feed was used. Water was boiled, cooled in sterile glass bottles and supplied freshly every day. The waterers were cleaned and rinsed with hot water (60°C) every day and then filled with sterile fresh cool water.

(v) Light and heat programme

For the first 24 hours after their arrival the chickens were given a 24 hours continuous light to allow them to have access to feed and water. Then the length of daylight was controlled by using a timer switch as described below.

Week	17	8.15 hours	Weeks	23	13.00 hours
,,	18	8.45 ,,	,,	24	14.00 ,,
,,	19	9.15 ,,	,,	25	14.30 ,,
,,	20	10.00 ,,	,,	26	15.00 ,,
,,	21	11.00 ,,	,,	27	15.30 ,,
,,	22	12.00 ,,	,,	28-End	16.00 ,,

Heat was supplied by heat bulbs and on cold days it was supplemented by the wall mounted electric heater. The temperature inside the unit varied between 12°C-22°C as shown by the maximum and minimum thermometer that was installed 0.75 m above the floor on the partitions between the 2 pens.

(vi) Health

All of the chickens were normal and no sign of any illness was observed at the time of delivery. Multivitamin drops (Abidec) were given at 7 drops per litre of drinking water for 3 days to reduce the stress of the journey. The vitamins were given before parenteral vaccination and routinely on 3 days each month to reduce the effects of the stress of bleeding.

At the age of 33 weeks, hen 3 in group 1 developed

bumblefoot which was treated by the intramuscular injection of 15000 IU of long acting penicillin (Duphapen LA, Duphar Veterinary Ltd., Gaters Hill, Southampton). The wound was also cleaned and disinfected locally.

The first treatment was successful and the hen was observed laying later on, but at the age of 47 weeks the problem recurred and then failed to respond to treatment. This time the general condition of the hen was poor and it was culled. Post mortem examination showed ascites, enlarged liver, involution of the oviduct and regression of ovarian follicles. The other chickens did not show any abnormality during the experiment.

(b) Vaccine preparation

Two types of Salmonella vaccine were prepared and used during this study. They were parenteral and oral vaccines and were prepared as follows.

(i) Parenteral vaccine

A loopful of organisms from sloppy agar was inoculated into nutrient broth and incubated in an orbital incubator at 37^{o}C for 16 hours at 140 rev/min. 3.5 ml of this broth culture was used to seed the surface of Roux flasks containing tryptone soya agar. The flasks were incubated at 37^{o}C for 30 hours (Thain et al. 1984). Salmonellae were harvested from the surface of culture medium in 10 ml volumes of sterile distilled water by gentle agitation. The process was repeated and the two suspensions combined. The number of bacteria in the suspension was counted by a Coulter Counter model Z.F. with C1000 channelyzer (Coulter Electronics, North Well Drive, Luton, Beds.) and adjusted to $1.4x10^{10}$ organisms per ml of suspension. 0.5% v/v formalin (BDH Chemicals Ltd., Poole, England) was added to the suspension and the mixture was kept at 4^{o}C. After every step of the culture process the organisms were checked

for purity and the final suspension was tested for sterility. There was no growth on the third day after the addition of formalin. Sterility examinations were continued daily until the sixth day after inactivation. After complete assurance of sterility, an oil emulsion vaccine was produced by mixing 3 ml of bacterial suspension, 6.75 ml of liquid paraffin (Riedel-De Haen A.G. Seelze, Hannover, Germany), 0.25 ml Arlacel^{R}A (Sigma), (Thain et al. 1984) and 0.3mg of surface protein antigen (described in Chapter 2, Section 2). All were mixed and emulsified using a laboratory mixer and emulsifier (Silverson Machines Ltd. Waterside, Chesham, Bucks.) at full speed while the container was kept in an ice bath. A few drops of vaccine were dropped into a beaker of cold water to check the state of emulsification with a syringe and needle. If the drops remained suspended intact in the water, the process was complete, otherwise emulsification was continued until stable submerged drops were formed in the water.

All the actions were carried out under sterile conditions. The product was checked for sterility and used as described below.

(ii) Oral vaccine

3.5 ml of a 16 hour nutrient broth culture was used to seed the surface of Roux flasks and these were incubated at 37^{o}C for 48 hours. The organisms were harvested in two 10 ml volumes of distilled water as described above and examined by culture for purity. The presence of flagella and fimbriae were confirmed by electron microscopy.

The bacterial suspension was formalised (0.5% v/v) and placed at 4^{o}C. Sterility was confirmed as described above. It was found that the bacterial suspension was so dense that the amount of formalin added had not sterilised the solution. The first batch was then placed in a water bath at 56^{o}C for one hour after sonication with an MSE 100 watt ultrasonic

disintegrator (Measuring and Scientific Equipment Ltd. London, England) for 15 minutes per 20 ml of vaccine. 0.75% formalin was added to the other batches before sonication. After completion sterility had been confirmed the suspension was aliquoted into universals and lyophilized (Truscott, 1981). The lyophilized vaccine was kept at $4^{o}C$ until used as described below.

(iii) Vaccination schedule

As explained above, the chickens were divided into 2 groups that were kept in the two separate parts of the isolated unit. From that time onwards they are referred to as groups 1 and 2. The hens of group 1 were parenterally vaccinated and both groups of chickens received oral vaccine.

Parenteral vaccine consisted of 0.5 ml amounts of the emulsion described above which was injected subcutaneously (Cameron and Fuls 1974, Germanier 1970) in the neck region (Thain et al. 1984). The first injection was given 3 weeks after the arrival of the chickens at 19 weeks of age and boosting doses were given at 23 and 36 weeks of age at the same sites with the same amount of freshly prepared vaccine.

Oral vaccine was first given at the age of 19 weeks at the rate of 1000 ppm of feed for 2 weeks, 500 ppm for another 2 weeks and finally at 250 ppm until the end of experiment (Truscott 1981).

The oral vaccine was first mixed with corn flour (Brown and Polson original patent corn flour) at a proportion of 1 in 20 and then mixed with the feed to ensure even distribution.

c) Samples

The following samples were taken from both groups to monitor the chicken's response to vaccination and their

Salmonella status.

(i) Cloacal swabs

The cloacas of all chickens were swabbed after arrival and then fortnightly using charcoal containing transwabs as described in chapter 2. After selective enrichment in tetrathionate broth every sample was subcultured onto DCA, SS and BG agar.

(ii) Faecal and litter samples

Pooled faecal samples were taken from each group every week by collecting 10 fresh droppings and mixing them in a sterile container using a sterile glass rod. 2 g of each sample was used to inoculate 20 ml of tetrathionate broth and after 24 and 48 hours incubation subcultured into SS, DCA and BG agar. Faeces were also cultured directly onto sheep blood, MacConkey and BG agar. Samples from litter were collected as described in Chapter 2 and cultured by the method described for faecal samples.

(iii) Blood samples

Blood samples were collected from the brachial vein and sera separated as described in Chapter 2, Section 4. Sera from each chicken were tested using the agglutination and ELISA tests.

Blood samples were obtained on arrival and then every fortnight, but to avoid stress effects on egg laying, the time interval between bleedings was increased to 3 weeks or one month during the laying period.

(iv) Eggs

Eggs were collected daily, cleaned and marked with the

date of lay and group of chickens and were then kept in 4°c until used in ELISA tests, hatching for the production of day old chicks or for the isolation of immunoglobulins from yolk and white.

(v) Oviductal washings and bile

At the end of the experiment both groups of chickens were killed and bile and oviductal washings were obtained as described in chapter 2. The immunoglobulin levels present in these samples was determined by the ELISA test.

(B) Results

a) Bacteriological studies

No Salmonellae were isolated from feed, water, litter, faeces or cloacal swabs during the whole period of the experiment. No other bacterial pathogens were isolated.

b) Egg production

Egg production began at the age of 24 weeks in group 1 (parenterally vaccinated). 4 days later the first egg was observed in group 2. First eggs were small. Shortly after lay began, production increased sharply to the peak level (80%) and continued at that level with a slight decrease (2%) for 29 weeks (time of killing of orally vaccinated group). Egg production and especially the size of the eggs and egg shell quality was affected by the necessity to bleed the hens at fortnightly intervals and the interval was therefore extended to 1 month.

Eggs weighing 55-65g with the average weight of 60g (Tullet and Noble 1988, Shanawany 1987) and suitable for hatching were obtained about 3 weeks after the start of laying.

c) Serological tests

(i) Agglutination test

All sera obtained from these chickens were screened by the agglutination test and the results are shown in Table 22. All chickens were negative prior to vaccination (week 17, first bleed), and the orally vaccinated ones of group 2 and group 1 (cockerel) remained so during the entire experiment. The parenterally and orally vaccinated hens were positive at the time of first post vaccinal bleeding (week 21) and remained positive during the experiment.

The effect of boosting doses of vaccine could not be observed by this test and the agglutinin antibody level did not decrease to the level below the sensitivity of this test in time intervals between vaccination (undiluted sera were used).

(ii) ELISA

All serum samples were serially diluted in the ELISA test described in Chapter 2, to determine the level of IgA, IgM and IgG against surface protein and LPS antigens of S. typhimurium pt. 49. IgM, IgG and IgA antibody levels were increased (Figs. 35-41 and Appendix 4) after parenteral vaccination. IgG had the highest titre among the three immunoglobulins (levels of 1:38400 to 1:76800, Figs. 35-36, Tables I-II in Appendix 4).

Antibodies were raised against both antigens in the same way but at different levels (Fig. 41). IgG titres ranged from 1:9600 (chicken 5, Table I) to 1:76800 (chicken 6, Table I) against surface protein antigen and from 1:4800 (chicken 5, Table II) to 1:38400 (chicken 6, Table II) against LPS antigen. After every vaccination the level of each immunoglobulin increased to a peak level and this reaction was the same for all three immunoglobulins.

Table 22

Results of agglutination tests on the sera of both groups of vaccinated chickens during Experiment 5.

Age in week	group 1 No. positive:No. birds	group 2 No. positive:No. birds
17	0:6*	0:6
21	5:6**	0:6
23	5:6	0:6
25	5:6	0:6
27	5:6	0:6
29	5:6	0:6
33	5:6	0:6
36	5:6	0:6
40	5:6	0:6
44	5:6	0:6
49	4:5***	0:6
52	---	0:6
59	4:5	---

* = Before vaccination.

** = 16 days after first vaccination.

*** = one hen from group 1 killed at week 47, all chickens in group 2 killed at week 52 and the rest at week 59.

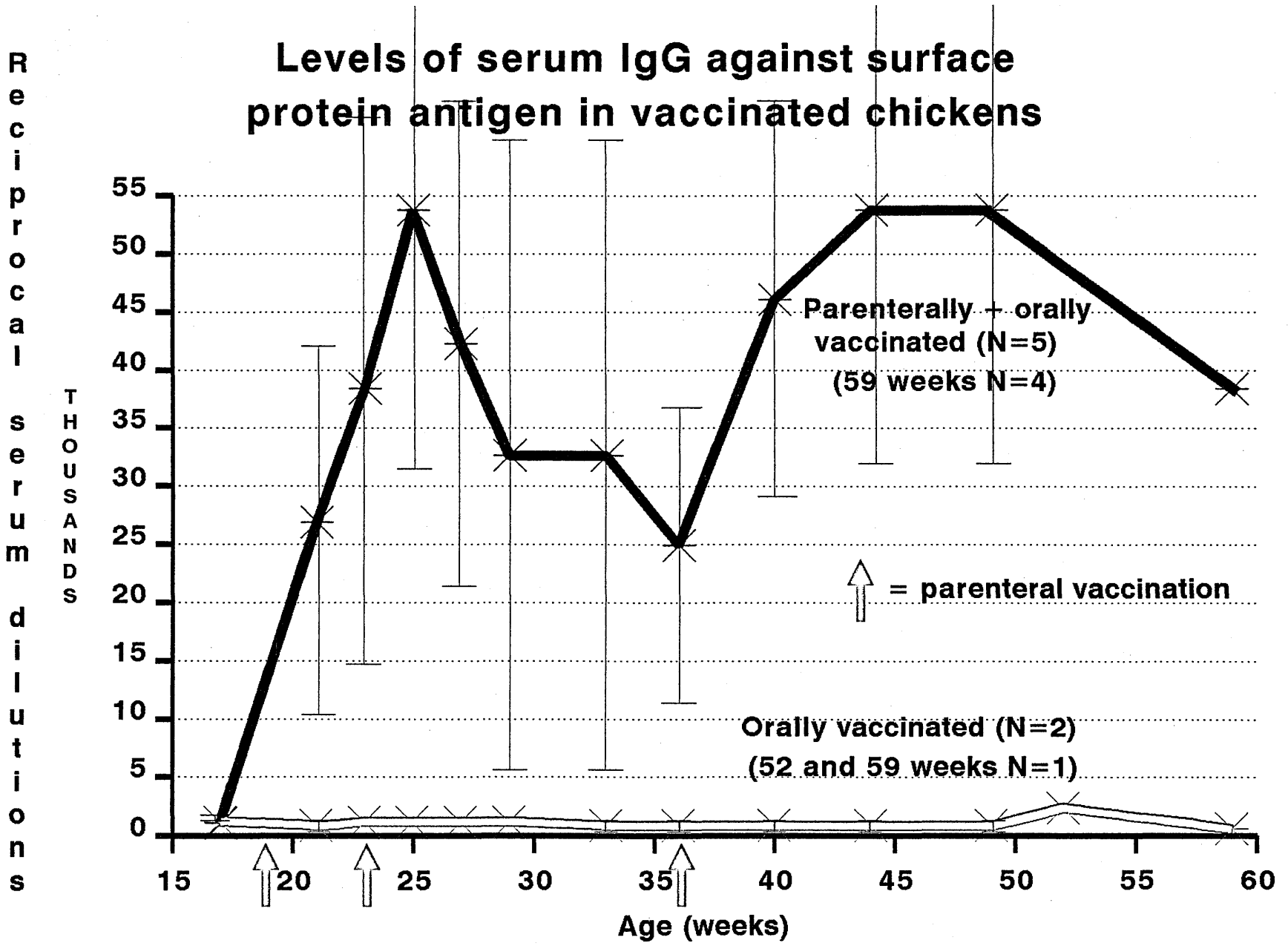
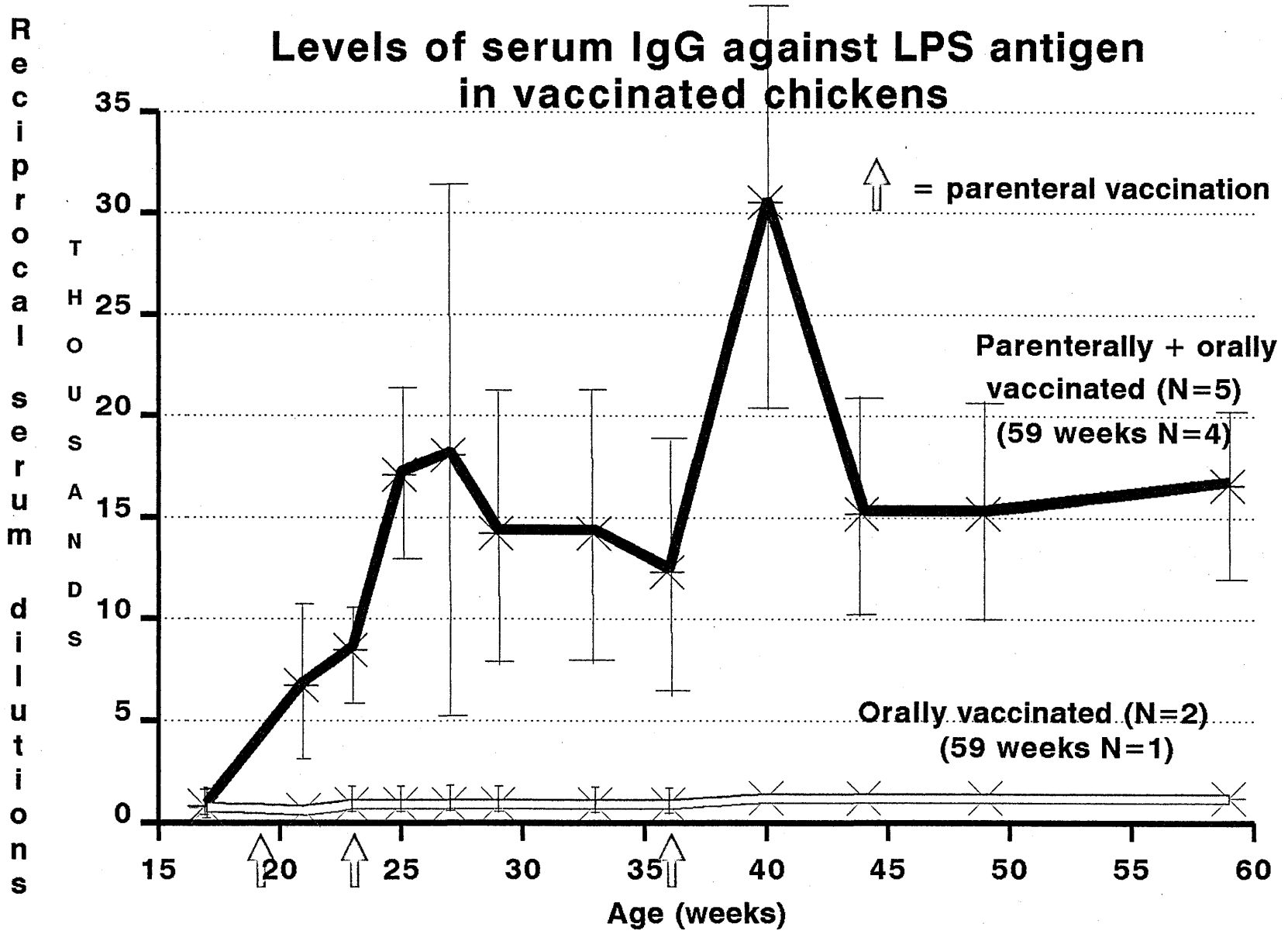
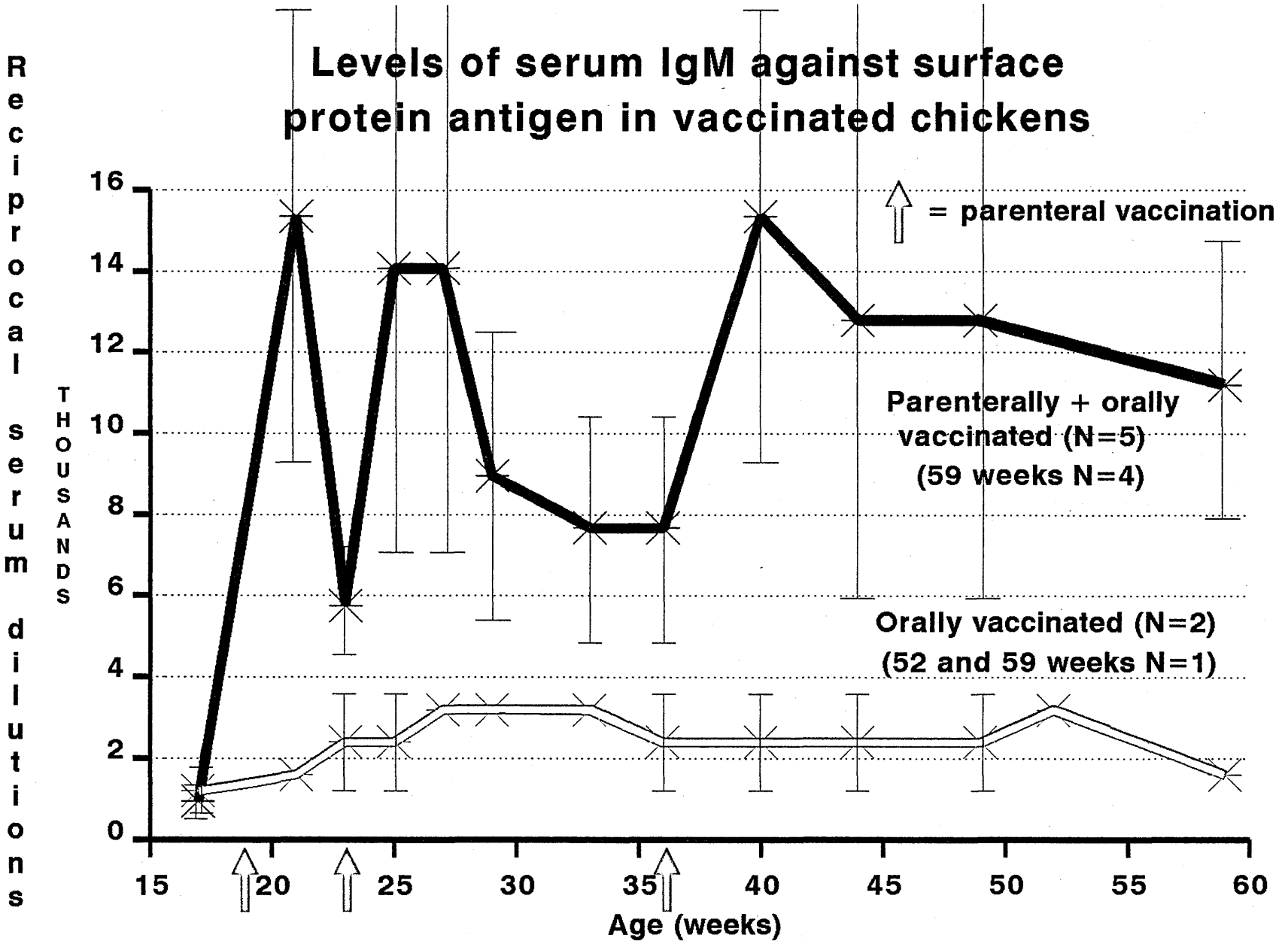

Levels of serum IgG against surface protein antigen in vaccinated chickens
Reciprocal serum dilutions
THOUSANDS
55
50
45
40
35
30
25
20
15
10
5
0
Parenterally + orally vaccinated (N=5)
(59 weeks N=4)
= parenteral vaccination
Orally vaccinated (N=2)
(52 and 59 weeks N=1)
15
20
25
30
35
40
45
50
55
60
Age (weeks)

FIG. 36:


Levels of serum IgG against LPS antigen
in vaccinated chickens
Reciprocal serum dilutions
THOUSANDS
35
30
25
20
15
10
5
0
= parenteral vaccination
Parenterally + orally
vaccinated (N=5)
(59 weeks N=4)
Orally vaccinated (N=2)
(59 weeks N=1)
15
20
25
30
35
40
45
50
55
60
Age (weeks)


Levels of serum IgM against surface protein antigen in vaccinated chickens
Reciprocal serum dilutions
THOUSANDS
16
14
12
10
8
6
4
2
0
15
20
25
30
35
40
45
50
55
60
Age (weeks)
= parenteral vaccination
Parenterally + orally vaccinated (N=5)
(59 weeks N=4)
Orally vaccinated (N=2)
(52 and 59 weeks N=1)

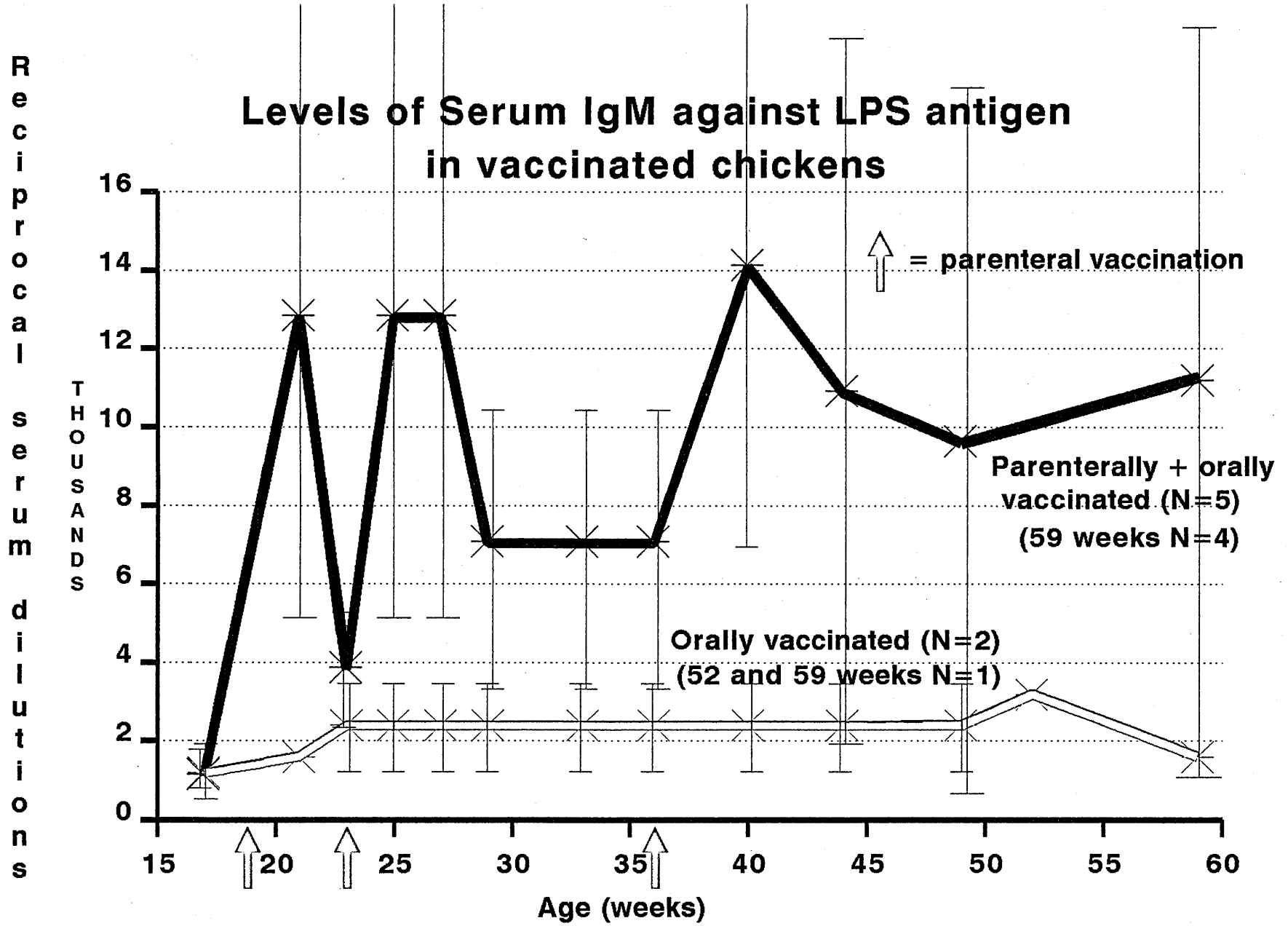

Levels of Serum IgM against LPS antigen
in vaccinated chickens
Reciprocal serum dilutions
THOUSANDS
16
14
12
10
8
6
4
2
0
15
20
25
30
35
40
45
50
55
60
Age (weeks)
= parenteral vaccination
Parenterally + orally
vaccinated (N=5)
(59 weeks N=4)
Orally vaccinated (N=2)
(52 and 59 weeks N=1)

FIG. 39:

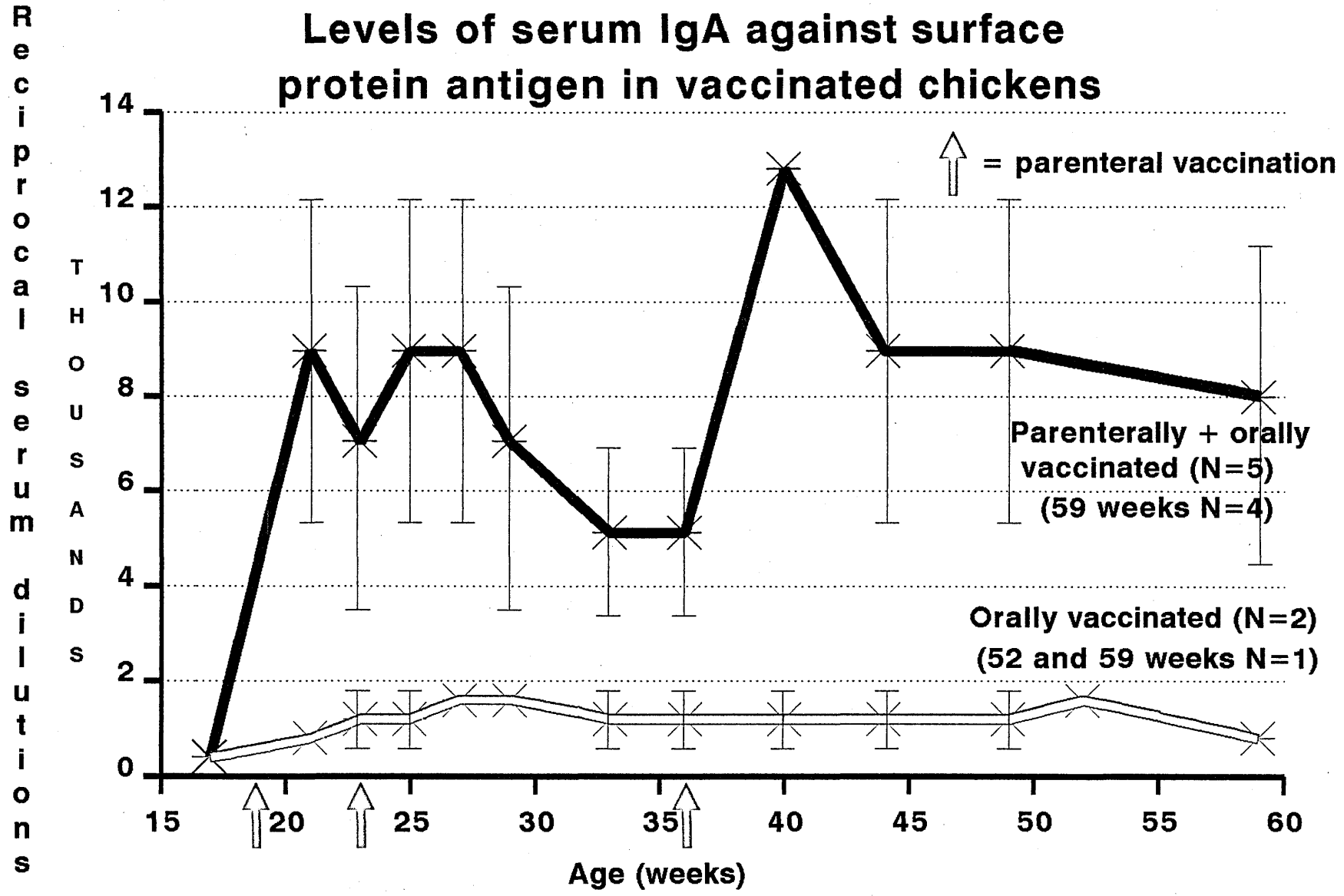

Levels of serum IgA against surface protein antigen in vaccinated chickens
Reciprocal serum dilutions
THOUSANDS
14
12
10
8
6
4
2
0
15
20
25
30
35
40
45
50
55
60
Age (weeks)
= parenteral vaccination
Parenterally + orally vaccinated (N=5)
(59 weeks N=4)
Orally vaccinated (N=2)
(52 and 59 weeks N=1)

FIG. 40:

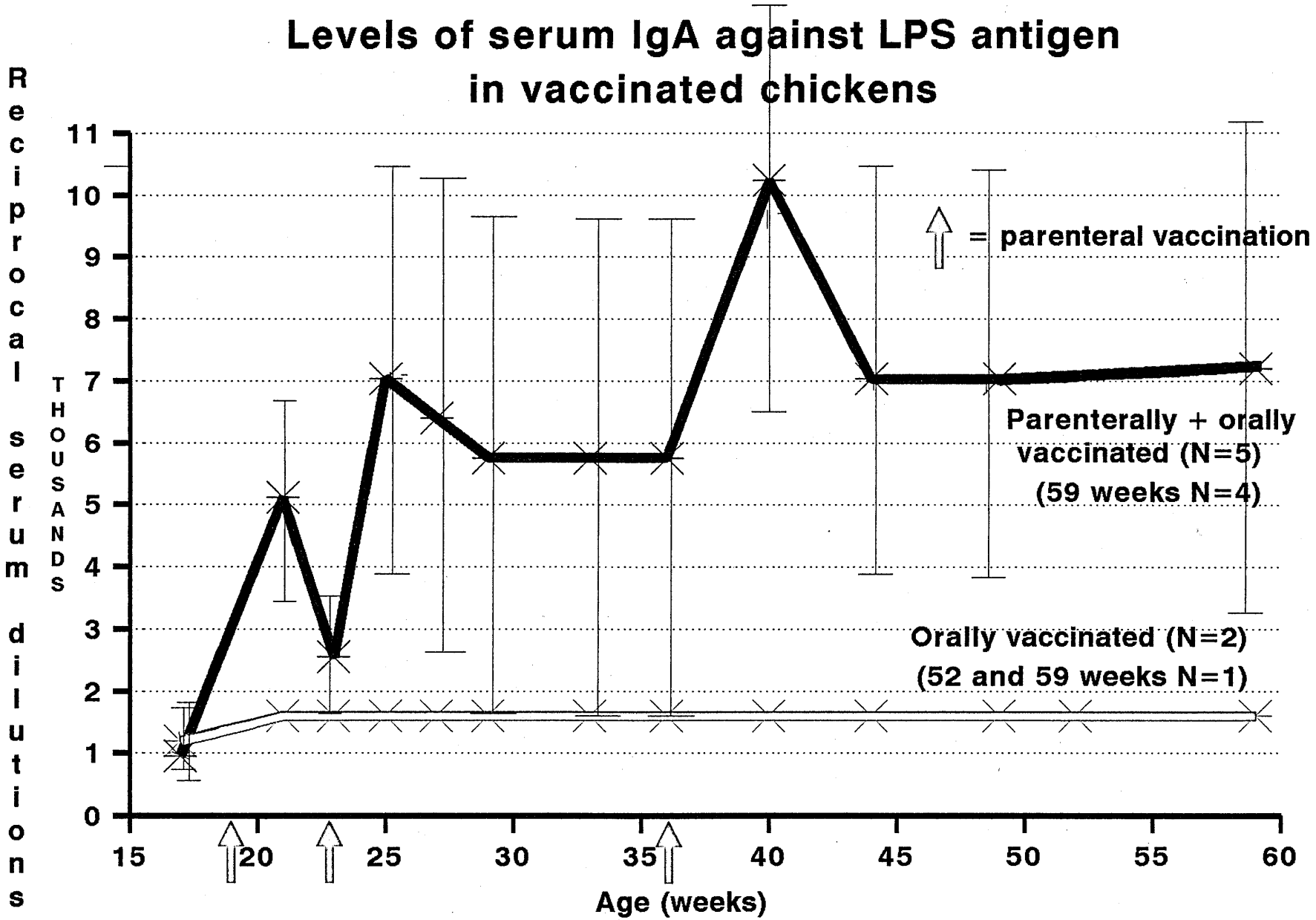
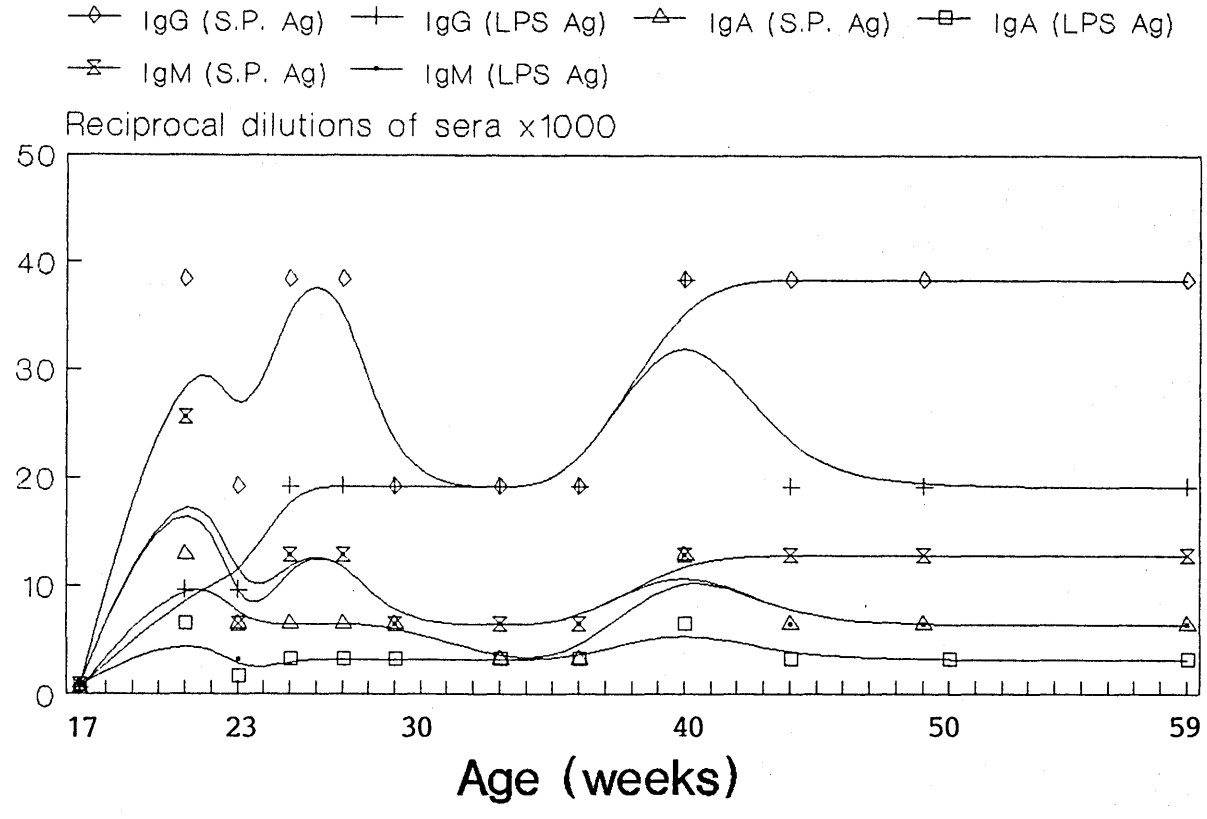

Levels of serum IgA against LPS antigen
in vaccinated chickens
Reciprocal serum dilutions
THOUSANDS
11
10
9
8
7
6
5
4
3
2
1
0
15
20
25
30
35
40
45
50
55
60
Age (weeks)
= parenteral vaccination
Parenterally + orally
vaccinated (N=5)
(59 weeks N=4)
Orally vaccinated (N=2)
(52 and 59 weeks N=1)

FIG. 41:

Levels of serum IgG, IgM and IgA against antigens of S. typhimurium pt. 49 in hen 2.



No IgG antibody was detected to S. typhimurium antigens in chickens 1 (male) and 7 (female) which only received oral vaccine (Figs. 35-36, Tables I and II). The initial levels of IgG in the SPF serum was 1:600 to 1:1200 against the surface protein antigen and 1:300 to 1:600 against LPS antigen. After vaccination it increased to 1:76800 (peak level) against surface protein antigen and 1:38400 against LPS (Figs. 35-36 and Tables I and II). The initial levels of IgM in the SPF chickens was 1:-800 to 1:1600 against both surface protein and LPS antigen. After parenteral vaccination, the peak level for this immunoglobulin was 1:25600 (chicken 6, Tables III and IV) against both antigens.

The level of IgA in the SPF sera was 1:400 against surface protein antigen and 1:800 to 1:1600 against LPS antigen (Table V-VI). Maximum titre for this antibody against both antigens was 1:12800 in group 1 (Figs. 39-40 and chickens 3 and 6, Tables V-VI). A slight increase in the levels of IgA and IgM was observed in the sera of chickens of group 2, but there was a marked difference between the levels reached in group 1 and those in the sera of chickens of group 2. Immunoglobulin production against LPS antigen was slower and lower in level when compared with that produced to surface protein antigen and especially so for IgG and IgA (Figs. 35-36 and 39-40).

d) Determination of immunoglobulin levels in other samples

(i) Eggs

At the time of first vaccination the hens were not in lay and laying began after second vaccination. Individual eggs laid by hens of group 1 show high level of IgG to both surface protein (1:40960) and LPS (1:20480) antigens (Table 23). Then after the eggs from the days that the chickens were in full lay (1 egg/hen) were screened in bulk. The results of bulk study on yolk showed IgG levels of 1:20480 to surface protein antigen (Fig. 42) and 1:10240 to LPS antigen. These levels decreased

Table 23

Levels of antibodies to S. typhimurium pt. 49 determined by ELISA in the individual eggs of the vaccinated chickens.

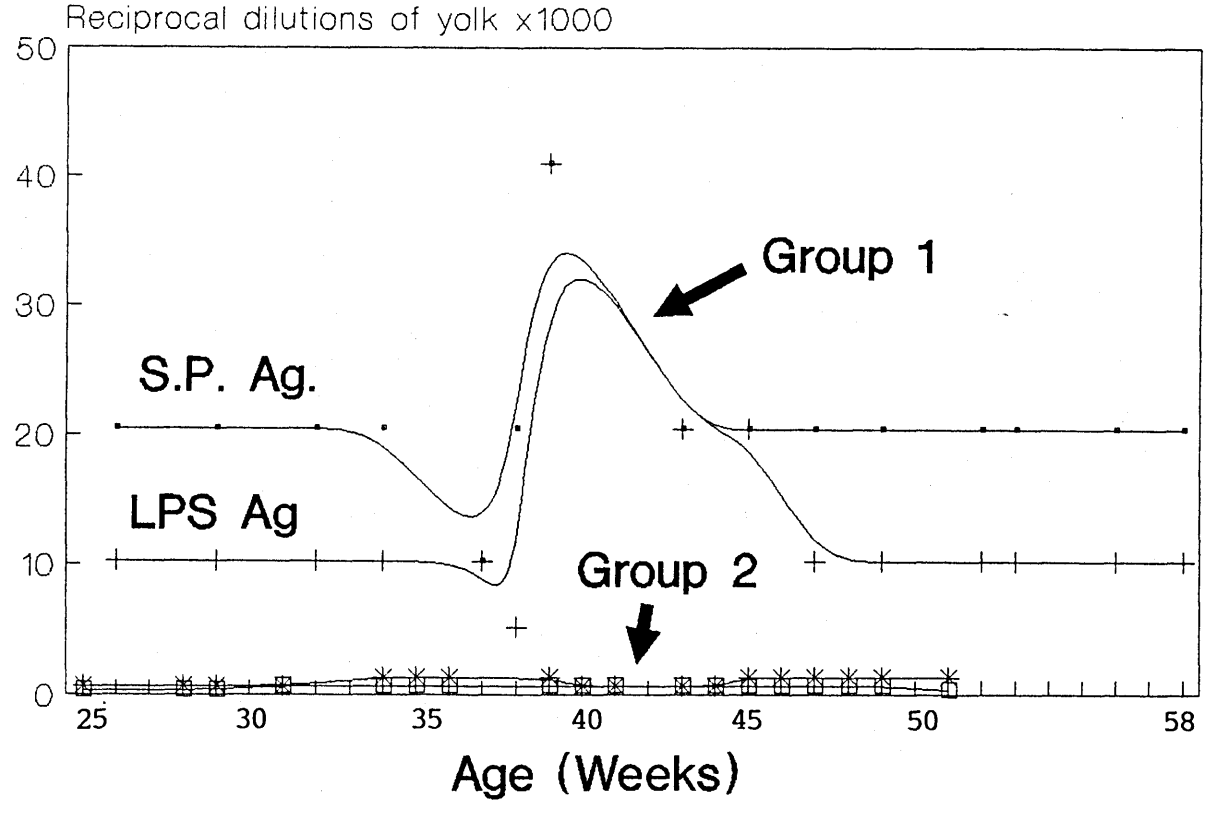
Type of Ig(s)	Group 1			Group 2		
	Age (weeks)	Titre		Age (weeks)	Titre	
		Protein Ag	LPS Ag		Protein Ag	LPS Ag
IgG	24	1:40960*	1:20480	25 A	1:2560	1:320
	27	1:40960	1:20480	25 B	N.D.	1:320
	59(2)+	1:20480	1:20480	52 A	1:2560	1:1280
	59(4)	1:40960	1:40960	52 B	1:2560	1:1280
	59(5)	1:10240	1:10240	52 C	1:1280	1:1280
	59(6)	1:40960	1:40960	52 D	1:1280	1:640
				52 E	1:640	1:320
IgA	24	1:320	1:640	25 A	1:160	1:320
	27	N.D.	1:640	25 B	N.D.	1:80
	59(6)	1:640	1:1280	52 A	1:320	1:160
				52 B	1:320	1:160
				52 C	1:320	1:160
				52 D	1:160	1:80
				52 E	1:320	1:320
IgM	24	1:320	1:640	25 A	1:160	1:320
	27	N.D.	1:320	25 B	N.D.	1:80
	59(6)	1:160	1:320	52 A	1:160	1:160
				52 B	1:320	1:160
				52 C	1:160	1:80
				52 D	1:80	1:160
				52 E	1:160	1:160

* = The end point dilution as determined by ELISA.

+ = The number inside the parenthesis indicates the number of the hen from which the sample was taken.

FIG. 42:

Levels of yolk IgG against antigens of S. typhimurium pt. 49 in the vaccinated chickens.



(one fold) prior to third vaccination and increased to a peak (1: 40960) against both antigens 3 weeks after the third vaccination. About 7 weeks later the titres fell by half and remained constant until the end of the experiment which was 23 weeks after the third vaccination.

The results of immunoglobulin studies on eggs are shown in Figs. 42-43 and Tables 23-24. 3 weeks after the third parenteral vaccination the level of IgG in the egg yolk of group 1 chickens increased to 1:40960 (Fig. 42) against both antigens. When pooled egg yolks were used, antibody titres to surface protein antigen ranging from 1:20480 to 1:40960 were observed and those against LPS antigen ranged from 1:5120 to 1:40960, during the experiment. In egg yolks from the hens of group 2, IgG antibody titres ranged from 1:640 to 1:1280 against surface protein antigen and 1:320 to 1:640 against LPS antigen. The results of studies on the individual eggs of group 1 hens are shown in Table 23. These egg yolks were obtained from the oviducts of the hens at the end of experiment. Hens 4 and 6 showed the highest titre (1:40960) and hen 5 the lowest titre (1:10240) against both antigens. Five eggs were laid by the hens in group 2 on the last day of experiment. Individual screening of these eggs showed the IgG level of 1:640 to 1:2560 against surface protein antigen and 1:320 to 1:1280 against LPS antigen (Table 23).

Bulk examination of egg white showed IgM levels of 1:80 to 1:160 against both antigens in groups 1 and 2 (Table 24). The last 5 eggs of group 2 that were individually examined, had titres of 1:80 to 1:320 against surface protein antigen and 1:80 to 1:160 against LPS antigen for IgM antibody (Table 23).

IgA levels were higher than those for IgM in the egg white of group 1 hens, with ranges of 1:160 to 1:320 and 1:320 to 1:640 against surface protein and LPS antigens respectively. After the third vaccination there was an increase in the level of IgA antibody against both antigens, while it was not

FIG. 43:

Levels of egg white IgA against antigens of S. typhimurium pt.49 in the vaccinated chickens.

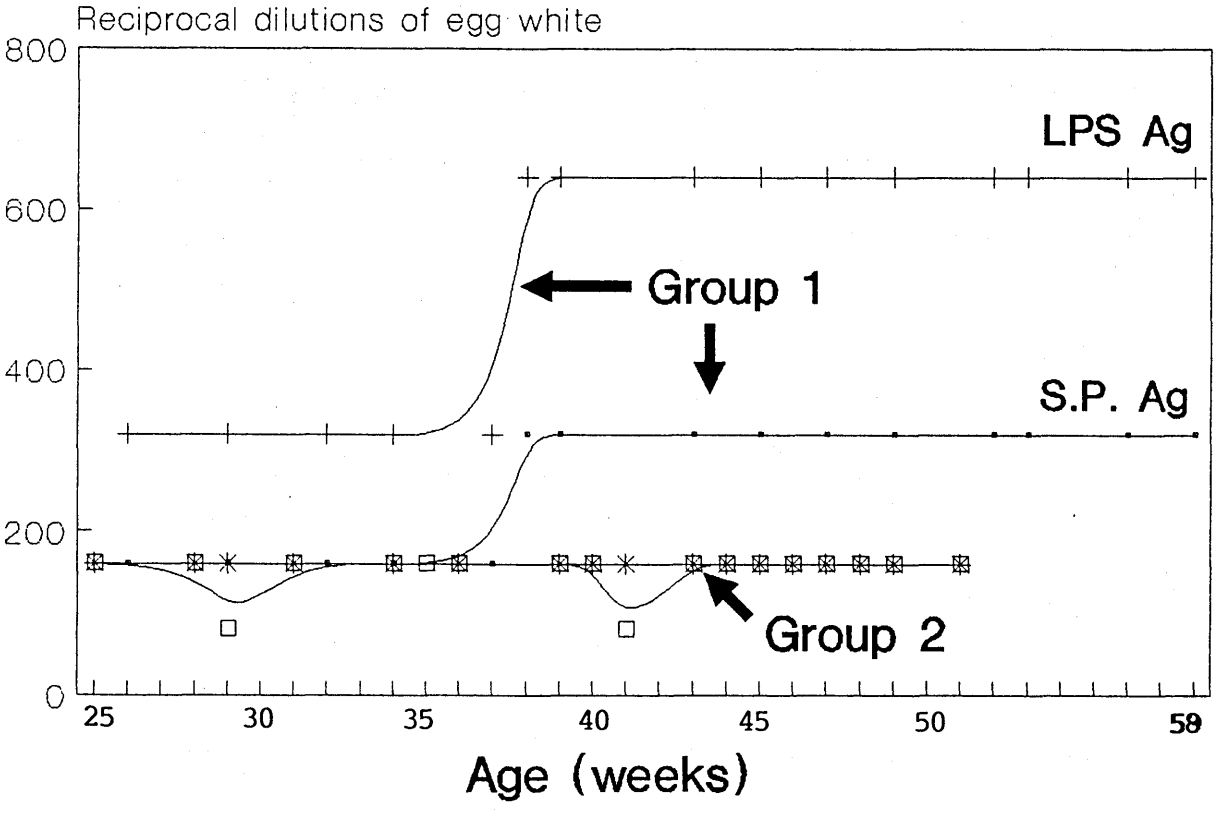


Table 24

Levels of IgM against surface protein and LPS antigens of S. typhimurium pt. 49, measured by ELISA in the bulk egg white of both vaccinated groups of chickens.

Age (weeks)	Group 1		Age (weeks)	Group 2	
	S.P. Ag*	LPS Ag		S.P. Ag	LPS Ag
26	80**	80	25	160	160
29	160	80	28	160	160
32	160	160	29	160	160
34	80	80	31	160	160
37	160	80	34	160	160
38	160	160	35	160	160
39	160	160	36	160	160
43	160	160	39	160	160
45	160	160	40	160	80
47	80	80	41	160	80
49	160	160	43	80	80
52	160	160	44	160	80
53	160	160	45	160	80
56	80	80	46	160	80
58	160	160	47	160	160
			48	160	160
			49	160	160
			51	160	80

* = Surface protein antigen.

** = Reciprocal dilutions of samples.

observed for IgM antibody. The IgA antibody levels in group 2 were 1:160 to surface protein antigen and 1:80 to 1:160 against LPS antigen (Fig. 43).

(ii) Oviductal secretions

There was no IgG in the oviductal secretions of both groups against either antigen of S. typhimurium pt.49 but IgA and IgM were both present. Individual variation was observed for both immunoglobulins and in both groups. In those of group 2 the IgA levels ranged from 1:32 to 1:64 against LPS and 1:32 to 1:128 against surface protein antigens (Table 25). The titres ranged from 1:32 to 1:512 and 1:64 to 1:512 against surface protein and LPS antigens respectively (Table 25) in group 1.

The level of IgM for each hen is also shown in Table 25. The ranges of 1:16 to 1:128 in group 2 against surface protein antigen and 1:8 to 1:64 against LPS antigen were observed in group 2 and those in group 1 were 1:32 to 1:256 against both antigens.

(iii) Bile

The levels of bile IgA and IgM determined by ELISA are shown in Table 26. There was individual variation in both immunoglobulin levels and the IgM levels were lower than IgA against both antigens.

3 - CHALLENGE STUDIES (EXPERIMENT 6)

A) Materials and methods

a) Eggs

20 SPF eggs, 44 eggs from orally vaccinated hens and 44 eggs from parenterally-orally vaccinated chickens were

Table 25

Levels of IgA and IgM antibody to S. typhimurium pt. 49 in the oviduct secretions from vaccinated hens as determined by ELISA.

Group	Hen No.	IgA		IgM	
		Protein Ag*	LPS Ag	Protein Ag	LPS Ag
1	2	1:32**	1:64	1:32	1:32
	4	1:64	1:128	1:64	1:64
	5	1:64	1:64	1:64	1:32
	6	1:512	1:512	1:256	1:256
2	7	1:64	1:64	1:64	1:64
	9	1:32	1:32	1:64	1:64
	10	1:64	1:32	1:64	1:32
	11	1:32	1:32	1:16	1:8
	12	1:128	1:64	1:128	1:64

* = Surface protein antigen of S. typhimurium pt. 49.

** = The end point dilution of samples as determined by ELISA.

Table 26

Bile IgA and IgM antibody to S. typhimurium pt.49 measured by ELISA in the vaccinated chickens.

Group	Bird No.	IgA		IgM	
		Protein Ag*	LPS Ag	Protein Ag	LPS Ag
1	1	1:640**	1:640	1:80	1:80
	2	1:640	1:640	1:80	1:80
	3	N.D.	N.D.	N.D.	N.D.
	4	1:640	1:640	1:160	1:160
	5	1:1280	1:1280	1:160	1:160
	6	1:1280	1:1280	1:640	1:640
2	7	1:640	1:640	1:320	1:160
	8	1:1280	1:1280	1:160	1:160
	9	1:640	1:640	1:160	1:160
	10	1:640	1:320	1:80	1:40
	11	1:640	1:320	1:320	1:160
	12	1:1280	1:640	1:640	1:160

* = Surface protein antigen of S. typhimurium pt. 49.

** = The end point dilution as determined by ELISA.

N.D. = Not done.

incubated to obtain chicks for this study. All eggs were in the range 56g to 65g. The eggs of vaccinated hens had been laid from the age of 39 weeks onward. All eggs were incubated within 10 days of laying. The incubators used and the conditions of incubation were described in Chapter 2, Section 3.

b)Types of experiment

The chicks derived from the 3 groups of eggs described above formed 3 experimental groups. Experimental groups 1 and 2 were derived from eggs of orally and parenterally-orally vaccinated hens respectively. Group 3 contained SPF chicks. In groups 1 and 2 there were 3 subgroups, A, B and C. The chicks in the A subgroups were killed at 4 hourly intervals after oral inoculation with the challenge organism and their organs were cultured, those of the B subgroups were exposed to 2 seeders and those of the C subgroups acted as negative controls. In group 3 there was only subgroup B as a control group for 1B and 2B. Every chick of the A subgroups and the seeders of the B subgroups were orally inoculated with 0.7×10^3 CFU of a fresh culture of S. typhimurium pt. 49 as described in Chapter 2, Section 3. Accommodation and feed were as described in Chapter 2, Section 3.

c) Bacteriological monitoring

All unhatched eggs and samples from the experimental groups were monitored for Salmonella infection by culture before and after inoculation or exposure. All chicks were swabbed prior to inoculation. The status of infection was assessed by culturing the organs of chicks in subgroups 1A and 2A at 4 hourly intervals and cloacal swabbing of the other chicks at daily intervals. 5 chicks from each of subgroups B and C were killed at 7 day intervals and their organs were cultured.

B) Results

a) Hatching

40 chicks were obtained from eggs from the orally vaccinated hens and 37 from eggs of the parenterally-orally vaccinated chickens. 16 chicks were hatched from the 20 SPF eggs that were used. 15 chicks of each group were placed in the B subgroups, 18 in 1A, 7 in 1C, 9 in 2A and 10 in 2C. The rest of the chicks were bled under terminal anaesthesia for serological studies.

b) Clinical observations

No signs of abnormality were observed prior to exposure. On the first day post exposure there was slight depression in subgroup 3B. On the second night post exposure, the brooder lamp of subgroup 2B burst, the temperature dropped and the chicks were huddling together next morning. On day 2 post exposure, depression, loose droppings and a decrease in feed consumption were observed and this continued for 4 to 5 days. The chicks in subgroups 1C and 2C did not show any signs of abnormality during the experiment and in comparison to these chicks the exposed chicks were retarded in their growth and used less feed and water.

On day 5 post exposure one of the chicks from subgroup 1B was in a moribund condition and was killed by Euthatal injection. Severe peritonitis and accumulations of caseous material were noticed in the abdomen. Salmonella and Staphylococci were isolated from its heart, liver, yolk sac and digestive tract.

c) Salmonella isolation

Salmonella was not isolated from unhatched eggs and embryos or from the cloacal swabs taken prior to inoculation or

exposure. Negative controls (groups 1C and 2C) did not show any sign of infection, neither by cloacal swabbing nor by the culture of their internal organs.

The chicks of group 2A derived from parenterally-orally vaccinated hens showed a delay of 4 hours or more in the colonisation of the gut (Table 27) when challenged by $0.7x10^3$ CFU of Salmonella. Under the same conditions and with an oral dose of $0.2x10^3$, 10^8 CFU of Salmonellae were cultured from the caecal contents of the SPF chicks in the first 4 hours post exposure (Table 27).

Salmonella was isolated from the caeca of most of the chicks and it was the only organ that was positive in all of the infected chicks (Tables 27-30). There was no systemic infection in the chicks derived from vaccinated hens which were monitored at 4 hour intervals (Table 28). The SPF chicks exposed to seeders were excreting the organism after 24 hours (3B, Table 31) and those derived from vaccinated hens after 48 hours (1B and 2B, Table 31).

The chicks derived from orally vaccinated and SPF groups showed systemic infection on day 7 post exposure, but those derived from orally-parenterally vaccinated hens did not show systemic infection at this time (Table 29). Salmonella was isolated from the heart and more frequently from the liver of systemically infected chicks. The unabsorbed yolk sac was infected with Salmonella in some chicks of all exposed groups. Crop, jejunum, ileum and caecum were colonised with the organism and the infection persisted up to 21 days (the end of the experiment, Table 29). The level of Salmonella in the caecal contents of exposed chicks was 10^9 CFU on day 7 post exposure and this decreased to 10^8 on day 14 and 10^6 on day 21 (Table 30).

Group 1B (derived from orally vaccinated hens) were infected with Enterobacter cloacae which interfered with the

Table 27

Salmonella counts from the caecal contents of chicks derived from orally vaccinated (1A) and Parenterally-orally vaccinated (2A) groups killed at 4 hourly intervals in Experiment 6.

Hours after infection	Chick No.	CFU of *Salmonella*/g of caecal contents		
		1A	2A	SPF*
4	1	0.00	0.00	6.33×10^8
	2	1×10^1	0.00	6.33×10^8
	3	1.33×10^6	0.00	2.66×10^8
8	1	1×10^9	1×10^8	1×10^9
	2	0.00	1×10^8	5.33×10^9
	3	6×10^8	1×10^9	3.66×10^9
12	1	1.33×10^8	2.66×10^9	4×10^9
	2	4×10^9	2.66×10^8	8.66×10^9
	3	3.33×10^9	3.00×10^9	6.66×10^9
16	1	7.00×10^9	---	4.00×10^9
	2	N.D.	---	3.33×10^9
	3	3.33×10^8	---	N.D.
20	1	3.66×10^8	---	7.66×10^9
	2	1.33×10^9	---	3.33×10^9
	3	6.00×10^9	---	N.D.
24	1	3.00×10^9	---	---
	2	4.66×10^9	---	---
	3	N.D.	---	---

CFU = Colony forming unit. N.D. = Not done.

* = These results were shown in Table 18 and are included here for ease of comparison. These SPF chicks were dosed with 0.2×10^3 CFU of *S. typhimurium* pt. 49.

Table 28

Salmonella isolation from the organs of chicks killed at 4 hourly intervals in the challenge study (Experiment 6).

Hours after infection	Organs cultured (1)	Subgroup 1A		Subgroup 2A	
		Direct (2)	Indirect(3)	Direct	Indirect
4	Heart	0:3*	0:3	0:3	0:3
	Liver	0:3	0:3	0:3	0:3
	Yolk sac	0:3	0:3	0:3	0:3
	Jejunum	0:3	0:3	0:3	0:3
	Ileum	0:3	0:3	0:3	0:3
	Caecum	1:3	1:3	0:3	2:3
	Crop	N.D.	1:3	0:3	0:3
8	Heart	0:3	0:3	0:3	0:3
	Liver	0:3	0:3	0:3	0:3
	Yolk sac	0:3	0:3	0:3	0:3
	Jejunum	0:3	0:3	0:3	0:3
	Ileum	0:3	0:3	0:3	1:3
	Caecum	2:3	2:3	3:3	3:3
	Crop	N.D.	1:3	0:3	0:3
12	Heart	0:3	0:3	0:3	0:3
	Liver	0:3	0:3	0:3	0:3
	Yolk sac	0:3	0:3	0:3	0:3
	Jejunum	0:3	0:3	0:3	0:3
	Ileum	0:3	0:3	1:3	1:3
	Caecum	3:3	3:3	3:3	3:3
	Crop	N.D.	1:3	1:3	1:3
16	Heart	0:3	0:3	---	---
	Liver	0:3	0:3	---	---
	Yolk sac	0:3	0:3	---	---
	Jejunum	0:3	0:3	---	---
	Ileum	0:3	0:3	---	---

Table 28 continued:

	Caecum	3:3	3:3	---	---
	Heart	0:3	0:3	---	---
	Liver	0:3	0:3	---	---
20	Yolk sac	1:3	1:3	---	---
	Jejunum	0:3	0:3	---	---
	Ileum	0:3	0:3	---	---
	Caecum	3:3	3:3	---	---
	Heart	0:3	0:3	---	---
	Liver	0:3	0:3	---	---
24	Yolk sac	1:3	0:3	---	---
	Jejunum	0:3	2:3	---	---
	Ileum	0:3	2:3	---	---
	Caecum	3:3	3:3	---	---

(1) = Salmonella was the only organism isolated from yolk sac as shown.

(2) = The samples were cultured directly onto sheep blood, MacConkey and BG agar without enrichment.

(3) = The samples were cultured into tetrathionate broth and then subcultured onto DCA, SS and BG agar.

* = No. of positives:Total No. cultured.

Table 29

Salmonella isolation from the organs of chicks in subgroups 1B, 2B and 3B killed at 7 day intervals in Experiment 6.

Days after exposure	Organs cultured (1)	1B		2B		3B	
		Direct (2)	In-direct+	Direct	In-direct	Direct	In-direct
7	Heart	0:5*	1:5	0:5	0:5	1:5	1:5
	Liver	1:5	3:5	0:5	0:5	4:5	5:5
	Yolk sac	1:1	1:5	2:2	2:2	N.D.	2:4
	Crop	0:5	2:5	2:5	4:5	4:5	5:5
	Jejunum	0:5	1:5	2:5	4:5	4:5	4:5
	Ileum	0:5	5:5	4:5	4:4	4:5	5:5
	Caecum	0:5	4:5	5:5	5:5	5:5	5:5
14	Heart	0:5	1:5	0:5	0:5	0:5	0:5
	Liver	0:5	0:5	0:5	1:5	0:5	1:5
	Yolk sac	N.D.	1:1	N.D.	2:3	N.D.	0:3
	Crop	0:5	4:5	2:5	3:5	5:5	5:5
	Jejunum	1:5	3:5	0:5	3:5	2:5	4:5
	Ileum	0:5	2:5	1:5	5:5	3:5	5:5
	Caecum	0:5	5:5	3:5	5:5	5:5	5:5
21	Heart	0:4**	0:4	0:5	0:5	0:5	0:5
	Liver	0:4	0:4	0:5	0:5	0:5	0:5
	Ileum	0:4	2:4	5:5	5:5	0:5	0:5
	Caecum	1:4	4:4	5:5	5:5	4:5	5:5

(1) = Salmonella was the only organism isolated from heart, liver and yolk sac as shown. (2) = The samples were directly cultured on sheep blood, MacConkey and BG agar without enrichment. + = The samples were cultured into tetrathionate broth and then subcultured onto DCA, SS and BG agar.

* = No. of positives:Total No. cultured.

** = One of the chicks died on day 5 post exposure.

Table 30

Salmonella counts from the caecal contents of chicks of all groups killed at 7 day intervals in Experiment 6.

Days after exposure	Chick No.	CFU of Salmonella/g of caecal contents		
		1B*	2B	3B
7	1	$< 10^8$	4.00×10^9	1.66×10^9
	2	$< 10^8$	3.33×10^8	3.00×10^9
	3	$< 10^9$	5.00×10^9	2.66×10^9
	4	$< 10^7$	1.00×10^9	6.66×10^8
	5	$> 10^7$	5.00×10^9	2.33×10^8
14	1	$< 10^7$	4.00×10^7	2.00×10^7
	2	$< 10^7$	1.67×10^8	1.00×10^7
	3	$< 10^7$	2.50×10^8	1.33×10^7
	4	$< 10^7$	2.00×10^8	1.00×10^7
	5	$< 10^7$	1.67×10^7	1.50×10^7
21	1	Not detectable	2.00×10^7	Not detectable
	2	,, ,,	1.00×10^7	5.00×10^6
	3	,, ,,	3.33×10^6	$< 5 \times 10^6$
	4	,, ,,	4.00×10^7	6.66×10^6
	5	---	1.00×10^7	1.50×10^7

* = Infection with Enterobacter cloacae interfered with Salmonella counts and due to heavy overgrowth of this organism the count was impossible on day 21.
CFU = Colony forming unit.

Table 31

Results of Salmonella isolation from cloacal swabs taken from subgroups B and C in Experiment 6.

Days after exposure	1B	2B	3B	1C	2C
0	0:15*	0:15	0:15	0:7	0:10
1	2:15	2:15	3:15	0:7	0:10
2	11:15	12:15	11:15	0:7	0:10
3	12:15	15:15	15:15	0:7	0:10
4	13:15	15:15	15:15	0:7	0:10
5	15:15	15:15	15:15	0:7	0:10
12	9:9	10:10	10:10	0:4	0:5

* = No. of positives:Total No. cultured.

growth of Salmonella on the plate by overgrowth. This problem made it impossible to count the Salmonella on day 21 post exposure (Table 30).

4 - DISCUSSION

The objectives of this study were:

a) To evaluate the ability of parenteral and oral Salmonella vaccines to stimulate the immune system of the chicken for antibody production.

b) To measure levels of specific antibody against S. typhimurium pt. 49 of each immunoglobulin class in the sera, bile and oviductal secretions.

c) To study the transfer of specific antibodies into the egg.

d) To study the role of these antibodies in the protection of young chicks against infection with the same organism.

A) The vaccination study

The health status of the host, freedom from infection, quality, potency, route of administration of the vaccine and host response to it are important factors that can affect the outcome of vaccination. These factors are discussed below in relation to the study carried out in this Chapter.

a) Maintenance of Salmonella freedom of birds

The measures taken to ensure freedom of the chickens from Salmonella infection appear to have succeeded. The birds were bought from a SPF flock which was free from the infections shown in Appendix 3. They were kept in an isolated unit (Fig. 32) from the time of delivery until the end of the experiment.

No Salmonellae or other bacterial pathogens were isolated from the chickens, litter, faeces or their feed and water. Egg laying was normal and no sign of any illness was observed either during the experiment or at the time of post mortem inspection except in hen 3 which developed bumblefoot and was culled. It is therefore reasonable to conclude that the chickens were healthy and free from pathogens especially Salmonella. Consequently the immunological responses in their sera, eggs and secretions must be considered to result from vaccination.

b) Quality of vaccines

Killed vaccine was used in this study for reasons which are discussed fully in Chapter 6. The vaccines were prepared so as to preserve the largest possible number of pathogenic determinants. The organism used was of the lowest possible passage number and attempts were made to enhance its content of surface protein antigens such as flagella and fimbriae. The E.M. studies showed that the organism was expressing both fimbriae and flagella when used for vaccine preparation.

To increase the potency of the parenteral vaccine a water in oil emulsion was made, using Arlacel A and paraffin (Thain et al. 1984, McKercher 1986). Arlacel A is mainly an emulsifying agent but it may also have an adjuvant effect on antibody formation as described by Berlin (1963). Oil-based adjuvants have been used since 1916, when lanolin and liquid paraffin were used to emulsify S. typhimurium vaccine for the vaccination of animals and it is reported that it improved the immune responses in vaccinated animals (McKercher 1986). At the present time the major poultry vaccines are formulated with oil adjuvants because they increase the protection and duration of immunity (Zanella and Marchi 1982).

The prepared vaccine was stable and visual examination of a few drops in tap water showed stable, submerged and intact

droplets of the finished vaccine which did not fade away for at least half an hour. No local reaction was observed after injection of the vaccine and it was presumed to be safe. No evidence of long term reaction was seen.

c) Potency of the vaccine

The potency or immunising capability of a killed vaccine depends on concentration of antigen(s), dosage, route of inoculation and response of the host.

A high number of organisms ($4.2x10^9$/ml of vaccine) was used in the parenteral vaccine to provide sufficient antigen to stimulate a high degree of protection and 0.03 mg/ml of surface protein antigen was added to enhance this further. The same was true of the oral antigen as the minimum dose found to be effective by Truscott (1981) was used.

d) The routes of administration

The primary aim was to produce hyperimmune hens so the oral and parenteral routes were combined for the administration of the sonicated lyophilized and oil-emulsion vaccines.

The effect of oral administration of Salmonella antigen in the feed of broiler flocks has been reported by Truscott (1981) to have had some degree of success. This type of vaccination has its prime effect on local antibody secreting cells in the gut or other organs of the chicken body such as oviduct and production of systemic antibody was not demonstrated to any great degree.

The subcutaneous area around the neck was chosen for the parenteral administration of the oil-emulsion vaccine because a) there were few blood vessels in the area b) ease of administration c) it had been used successfully by other research workers in the turkey (Thain et al. 1984), in chickens

and mice (Knivett and Stevens 1971).

e) Results of vaccination

The ELISA was used successfully to demonstrate the changes in the different immunoglobulin classes in the serum of vaccinates. Parenteral vaccine was able to produce a systemic reaction but oral vaccine failed. The details are discussed below.

f) The vaccine programme

The main aim of this vaccination study was to provide protective immunity to the young chicks. The hens were therefore vaccinated prior to the commencement of lay in order to have circulating antibodies at the time of development of the yolk and possibly to prime the developing oviduct with IgA and IgM secreting cells producing specific antibodies against Salmonella antigens. The levels of circulatory antibodies decreased with age. Booster injections of parenteral vaccine were carried out to increase and maintain their levels. These were successful (Figs. 35-41) and maintained high serum levels in all hens until the end of the study. The oral vaccine was supplied continuously to maintain its local stimulatory effect, but had little effect on serum antibody levels as expected.

B) The antibody study

Parenteral vaccination stimulated the humoral immune system of the hens to produce specific antibodies against surface protein and LPS antigens of S. typhimurium pt. 49 both generally (systemically) and locally. Oral vaccination had a local effect only. The findings in serum, bile and oviductal secretions are discussed below.

a) Serum

The chickens that received oral vaccine alone failed to produce any measurable systemic IgG, IgA or IgM. The sera of these chickens were negative in agglutination test and did not show any increase in IgG antibody titres by ELISA. The level of IgG against Salmonella was constant and the same as that at delivery time. In the serum of one orally vaccinated chicken (hen 7) IgA and IgM showed a two fold increase when compared to the SPF sera examined at the time of first bleeding. The reason for this change is not clear since there was no increase in IgG levels and the level remained constant throughout the experiment. The parenterally-orally vaccinated chickens produced high levels of IgM, IgG and IgA after vaccination. The response of individuals to the vaccine varied but all showed the same pattern of increase and decrease in the antibody titres. The use of control pre inoculation sera assured the specificity of the reaction.

Titres of 1:76800 for IgG (Table I, hens 3 and 6), 1:25600 for IgM (Table III-IV, hens 2 and 6) and 1:12800 for IgA (Table V-IV hens 3 and 6) against surface protein antigen were observed. Higher titres were observed against surface protein antigen when compared to LPS antigen. IgG had the highest titre followed by IgM and then IgA. Despite the variation among the individuals the antibody titres were always higher than in the orally vaccinated group and marked differences between the groups were maintained during the entire experiment. The effect of the boosting vaccination on antibody production (anamnestic effect) was quite clear. Levels of all 3 immunoglobulins were increased after the second and third injections. The sera of parenterally vaccinated hens were positive by the agglutination test throughout the entire experiment and changes in antibody titres demonstrated by the ELISA did not affect the performance of this test.

These findings suggest that oral vaccine has no value for

systemic protection of vaccinated chickens due to its failure to stimulate systemic antibody production. Parenteral vaccination resulted in antibody production and high antibody levels were observed in all immunised chickens. At this stage it can be concluded that the parenteral vaccine was potent and that the high levels of antibodies produced can be transferred to the eggs and may play a part in the protection of young chicks. The part played by simultaneous oral vaccination in supplementing the levels of immunity stimulated by parenteral vaccine could be assessed.

b) Bile

Both IgA and IgM specific antibody was recognised by the ELISA in the bile of both groups of vaccinated chickens (Table 26). In all chickens these titres were lower than those in the serum of the relevant chickens. The ranges observed for IgA (1:320 to 1:1280) were lower than those of naturally infected chickens (1:1280 to 1:10240) reported in Chapter 3, but higher than those of the SPF chickens (1:80 to 1:320, Table 14).

IgM titres were lower than IgA and within the same range in the bile of both vaccinated groups and naturally infected chickens (1:40 to 1:640) and slightly higher than those in SPF chickens (1:40 to 1:80, Table 14). There is no doubt about the presence of specific IgA and IgM in the bile. The 6 culled SPF hens sampled as controls in this study did not have access to feed and water for at least 24 hours before killing because they were on the way to the Veterinary School. It means that their bile was highly concentrated as observed at the time of sampling. Despite this the levels of immunoglobulins in the bile of experimental chickens were higher. All bile samples were collected from the gall bladder and it is possible that they might have been subjected to loss of water and concentration to different degrees in different individuals. Thicker and thinner bile solutions were observed at the time of sampling. Due to this effect the results obtained might be

inconclusive and to have valid results for better comparison or for diagnostic purposes, bile samples must be collected by direct cannulation from the hepatic bile ducts rather than from the gall bladder. As this was not the main aim of this experiment no further investigation was conducted for a conclusive result. No IgG was isolated from bile.

c) Oviductal secretions

IgA and IgM were present in the oviductal secretions. The titres of these two immunoglobulins showed a higher range in parenterally-orally vaccinated hens than in orally vaccinated hens (Table 25) and in both groups were higher than those of SPF hens (Table 15). The presence of specific antibodies in both groups suggests that both types of vaccine regime may have been capable of stimulating local antibody production in the oviduct. This phenomenon would be expected for parenterally vaccinated hens with respect to the present knowledge of the avian immune system. However, more investigation is necessary to find out the mechanism of this stimulation by the oral vaccine which failed to produce a systemic reaction.

The results proved the ability of the parenteral vaccine to stimulate systemic and local antibody production. After vaccination the levels of these antibodies remained high in the sera (Figs. 35-41) for at least 23 weeks which could be due to the effect of adjuvant. With the advantages of long lasting immunity and no local reaction in the area of injection this vaccine represents a potential vaccine for the vaccination of parent flocks for protection of young chicks against Salmonella infection. The oral vaccine may be useful for increasing the local defence capability as claimed by Truscott (1981) and it may also have a synergistic effect in conjunction with parenteral vaccine as shown by Sow Intagen, BOCM in pigs. As the parenteral vaccine increases the antibody titre, further investigation is necessary to determine any possible cross reaction of these antibodies with the pullorum test if it is

going to be used for vaccination of commercial flocks.

C) Immunoglobulin transfer to the eggs

The results clearly show that egg yolk IgG levels are dependent on those in the serum and changes in yolk IgG levels (Fig. 42) reflects the changes in the serum (Figs. 35-36). The time delay of about maximum 3 weeks for peak can be accounted for by IgG production in serum and its transfer to the yolk. The level of IgG to LPS was lower than that to surface protein antigen in both bulk samples and individual screening tests. The results obtained on the individual yolks collected at slaughter show a variation among the individuals which is correlated to the serum IgG levels of the relevant hens. All hens in group 1 had high titres of IgG (minimum of 1:10240) to both antigens (Table 23).

IgA and IgM were found in the egg white and not in the egg yolk. Bulk studies show much lower IgA titres in eggs obtained from hens in group 2 than those in eggs of group 1. No correlation was found between the levels of this antibody with that in serum. For example, during the entire period of the experiment, IgA levels followed a linear pattern against both antigens doubling in the week after third vaccination. This increase might reflect the effect of vaccination on local antibody secreting cells in the oviduct, possibly by increasing their populations.

No such a change was observed in group 2 (orally vaccinated) and the titres against both antigens were constant. The fluctuation in the levels of IgA against LPS antigen in weeks 29 and 41 might reflect the effect of combining eggs of different sizes or an error in the test. If the rate of antibody production by the secretory cells is constant, the changes in egg size during each clutch period may cause variation of the antibody level in the egg white.

IgM levels ranged from 1:80 to 1:160 against both antigens and in both groups of vaccinated hens (Table 24). There were two fold fluctuations in the levels of these immunoglobulins at different time intervals and the levels did not appear to be affected by the third vaccination in vaccinated group 1. The reason for these changes could be the same as those described above for IgA in the eggs of orally vaccinated hens. The eggs from group 2 that were individually screened (Table 23) show the range of 1:80 to 1:320 for IgA and IgM and this range is the same for those eggs in weeks 25 and 52 (the day before slaughter).

The results suggest that IgA and IgM are present in the egg white of both vaccinated groups and their levels are lower than IgG in the yolk. Boosting vaccination did not increase the level of IgM. The titres of IgA and IgM in the egg white of orally vaccinated chickens were lower than those in the parenterally-orally vaccinated hens but constant during the entire period of the experiment.

D) Challenge study

The high percentage hatch and failure to isolate Salmonella and other pathogenic bacteria was the result of using clean and fresh eggs and the employment of rigorous hygiene in the incubation processes. No Salmonella was isolated from hatched chicks prior to experimental infection or exposure to infection. No systemic infection was observed in the chicks that were monitored at 4 hourly intervals and no Salmonellae were counted from the caeca of these chicks in group 2A in the first 4 hours after infection. After colonisation of the gut, Salmonella levels were the same in both groups. The caecum was the only organ that was always positive for Salmonellae isolation during the 24 hour period of the experiment (Table 28).

In groups of exposed chicks, Salmonella shedding was first

observed in the SPF group (seeders excluded) on day 1 post exposure and on day 2 in all 3 groups (Table 31). A slight difference was observed between chicks in group 1B and those in the SPF group as some chicks resisted the infection until day 4. Chicks in group 2B (derived from parenterally-orally vaccinated hens) acquired the infection at the same rate as SPF ones. This is possibly due to the effect of cold stress on night 2 post exposure. Soerjadi et al. (1979) showed that cold stress in day old chicks increased the number of birds shedding S. typhimurium. There was a difference in colonisation of the gut between these chicks with those derived from orally vaccinated and SPF hens and no systemic infection was observed on day 7 post exposure in contrast to the other 2 groups which did show systemic infection. It was planned to investigate further the role of each immunoglobulin in protection against infection. For this reason, this experiment was not repeated.

The results of Salmonella counts from caecal contents of the exposed chicks did not show any difference between the groups on days 7, 14 and 21 post exposure. The chicks derived from orally vaccinated hens were infected with Enterobacter cloacae at some stage after the exposure of chicks to Salmonella. This bacteria was over grew the plates and prevented accurate Salmonella counting. The chicks that were kept as negative controls did not shed any Salmonella in the period of experiment. These chicks were not weighed, but visual comparison with the infected ones showed increased size, better feathering and growth which indicates that Salmonella infection was suppressing the growth of the infected chicks.

CHAPTER 5

PASSIVE IMMUNOGLOBULIN TRANSFER IN THE PROTECTION OF THE NEWLY HATCHED CHICKS AGAINST CHALLENGE WITH SALMONELLAE

1 - INTRODUCTION

The results of the studies presented in chapter 4 indicated that the parenteral vaccine used was able to stimulate the immune system of chickens and produce antibodies against the surface protein and LPS antigens of S. typhimurium pt.49. Antibodies were demonstrated in serum, bile and oviductal secretions. Also it was demonstrated that specific IgG, IgA and IgM were transferred into the eggs of vaccinated chickens and from the evidence presented, the role of these immunoglobulins in the protection of chicks in early stage of life could be postulated.

In this study, attempts were made to determine the presence and persistence of passively transferred antibodies in the chick's sera. Immunoglobulins isolated and purified from egg yolk (IgG) and egg white (IgA+IgM) were injected into the yolk, albumin and allantoic cavity of SPF eggs. Eggs with passively transferred antibodies were incubated and the resultant chicks were used in the challenge study to determine the role of passively transferred immunoglobulins in the protection of young chicks against infection with S. typhimurium pt. 49.

2 - THE DEMONSTRATION OF PASSIVELY TRANSFERRED IMMUNOGLOBULINS IN CHICK SERA (EXPERIMENT 7)

A) Materials and methods

Day old chicks were obtained from the eggs of both vaccinated groups of chickens described in Chapter 4. The eggs used were laid at age of 43 weeks onward. Incubators, feed and

pens were described in Chapter 2, Section 3.

The chicks were bled just after hatch (Day 0)and then every 5 days up to 30 days of age. Blood was collected from the heart and sera were separated as described in Chapter 2, Section 4.

Each serum sample was screened by the agglutination test immediately after preparation and the rest of it was kept at -20°C until examined by the ELISA for the presence of specific IgG, IgA and IgM against the antigens of S. typhimurium pt. 49. Sera of the chicks derived from naturally infected hens (described in Chapter 3) and from SPF eggs were examined under the same conditions by the agglutination test and the ELISA.

B) Results

No agglutinating antibody could be demonstrated in the serum of chicks derived from the orally vaccinated hens, in the SPF chicks nor in the chicks derived from naturally infected hens when screened using the agglutination test on undiluted sera (Table 32).

All sera of the chicks derived from parenterally-orally vaccinated hens were positive up to 10 days of age by the agglutination test. On day 15 only one of four sera examined was positive and from then on, all sera examined were negative (Table 32).

The levels of specific immunoglobulin measured by the ELISA are shown in Table 33. A level of 1:38400 was observed for IgG against surface protein antigen of S. typhimurium pt. 49, in the serum of recently hatched chicks derived from parenterally-orally vaccinated hens. Under the same conditions this level was 1:2400 for chicks derived from orally vaccinated hens and from SPF eggs. 2 chicks derived from naturally infected hens had titres of 1:4800 and one had a

Table 32

Results of the agglutination tests on the sera of chicks derived from parenterally-orally vaccinated (group 1), orally vaccinated (group 2), naturally infected (group 3) and SPF hens in Experiment 7.

Age(days)	Group 1	Group 2	Group 3	SPF
0*	6:6**	0:3	0:3	0:3
1	8:8	---	---	---
5	4:4	0:3	---	0:5
7	---	---	0:5***	---
10	4:4	0:3	---	---
15	1:4	0:3	---	---
20	0:3	0:3	---	---
25	0:5	0:5	---	---
30	0:4	0:4	---	---

* = Immediately after hatching.

** = No. of positives:Total No. of sera examined.

*** = Done on the sera of the negative control chicks of Experiment 4.

Table 33

Levels of IgA, IgM and IgG against antigens of S. typhimurium pt.49 in the serum of chicks derived from vaccinated hens, naturally infected hens and SPF eggs, measured by ELISA in Experiment 7.

Chicks* derived from	Age (day)	IgG		IgM		IgA	
		S.P.Ag+	LPS Ag	S.P.Ag	LPS Ag	S.P.Ag	LPS Ag
	0	38400**	9600	1600	800	3200	1600
Parenterally	5	38400	4800	1600	400	3200	800
-orally	10	19200	2400	800	400	1600	800
vaccinated	15	9600	1200	800	200	1600	400
hens.	20	4800	600	200	100	400	200
	25	2400	600	100	100	200	100
	30	1200	300	100	100	100	100
SPF eggs	0	2400	1200	200	200	400	400
Orally vaccinated hens	0	2400	1200	400	200	400	400
	0	2400	1200	200	200	400	200
Naturally infected hens.	0	4800	600	200	100	400	200
	0	4800	1200	400	200	400	400
	0	2400	1200	100	100	200	100

* = All figures represent the measurement on the serum of individual chicks except that pooled sera was used from SPF chicks.

+ = Surface protein antigen.

** = Reciprocal dilutions of sera.

titre of 1:2400 for IgG measured against this antigen. Specific IgA (1:3200 against surface protein and 1:1600 against LPS antigens.) and IgM (1:1600 against surface protein and 1:800 against LPS antigens) were detected in the sera of chicks derived from the parenterally-orally vaccinated hens (Day 0, Table 33). Lower levels were detected in the sera of the other groups. The immunoglobulin levels in the sera of all four groups on day 0 are compared in Fig. 44. There is a marked difference between the level of immunoglobulin in the sera of chicks derived from the parenterally-orally vaccinated chickens and those of other groups. In all cases lower antibody levels were observed against LPS antigens than against surface protein antigen. The levels of these immunoglobulins declined and were undetectable by the agglutination test in the sera of 15 day old chicks (Table 32). The decline as measured by the ELISA is shown in Table 33 and is illustrated for IgG against both antigens in Fig. 45. Sera used at each time interval were from chicks other than those examined previously, but levels of IgG fell by half every five days (Table 33).

3 - PROTECTION STUDIES (EXPERIMENTS 8, 9 AND 10)

A) Materials and methods

a) Isolation and purification of egg immunoglobulins

To examine the role of each immunoglobulin in the protection of the chick against infection with S. typhimurium pt. 49, IgG and IgA+IgM were isolated from the yolk and the white of the eggs of parenterally-orally vaccinated hens, purified and injected into SPF eggs. The chicks hatched from these eggs were challenged with the organism.

(i) Isolation of IgG from egg yolk

IgG was isolated from egg yolk by a modification of the methods described by Jensenius et al. (1981) and Johnstone and Thorpe

FIG. 44:

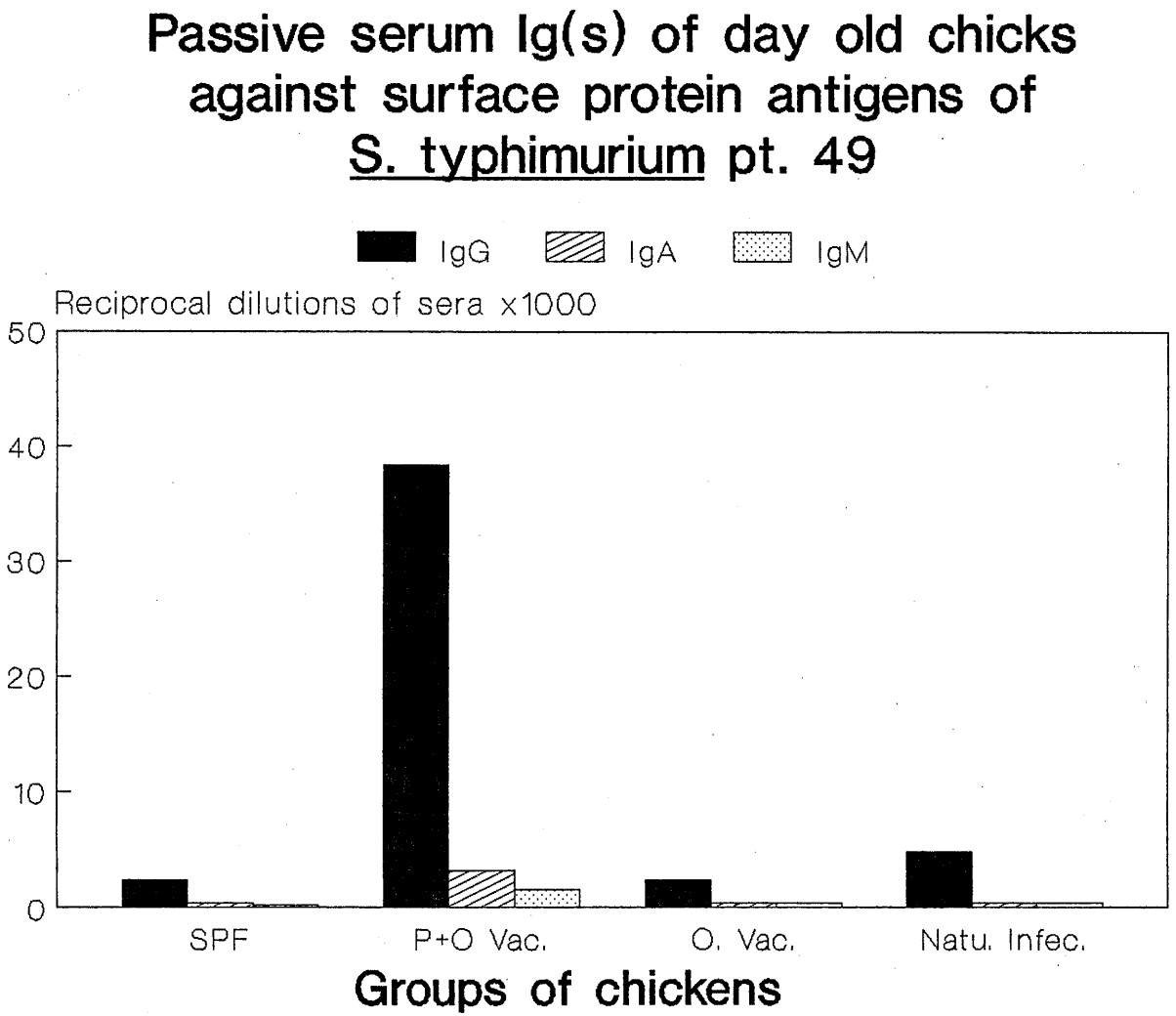
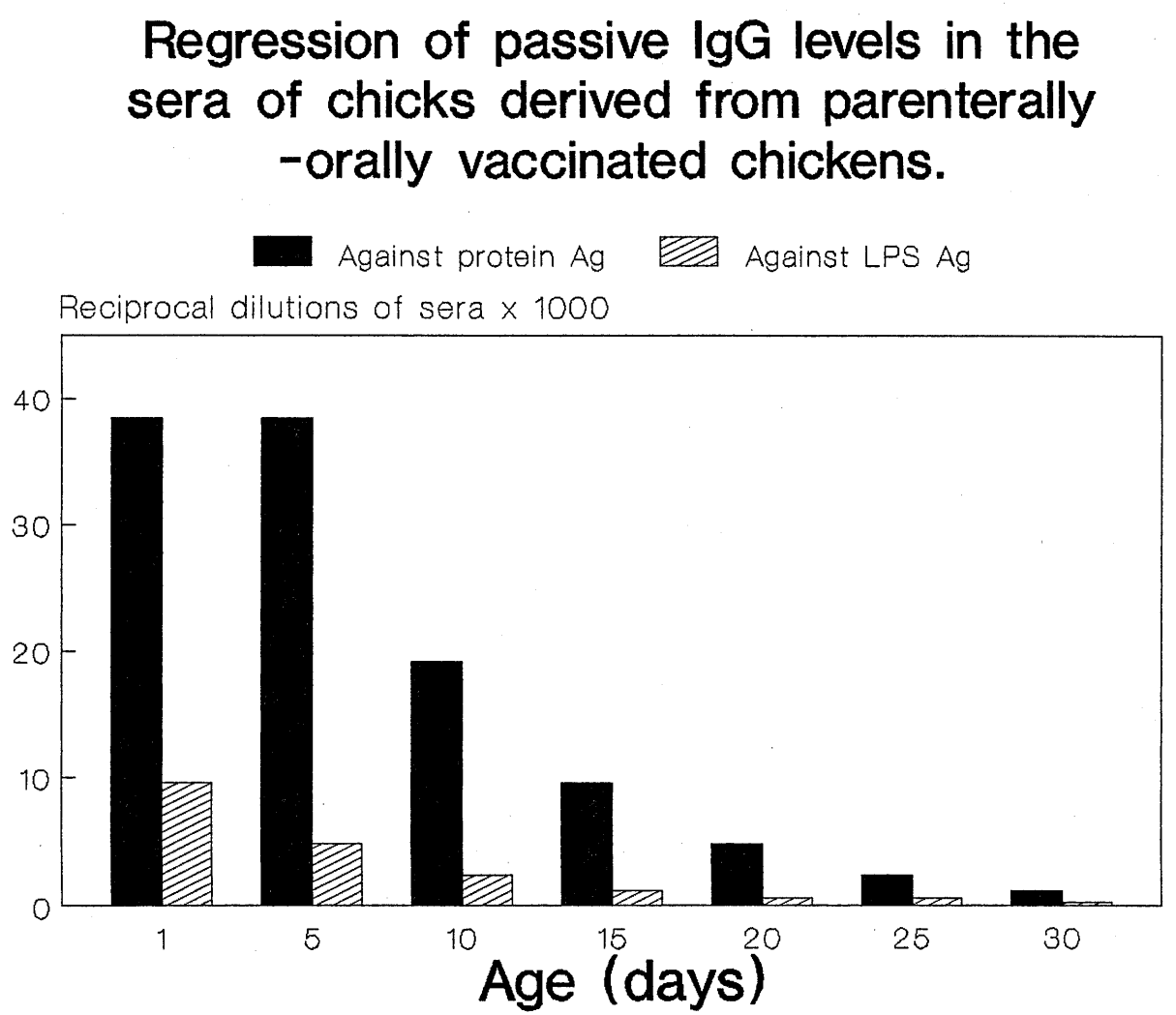

Passive serum Ig(s) of day old chicks against surface protein antigens of S. typhimurium pt. 49
IgG
IgA
IgM
Reciprocal dilutions of sera x1000
50
40
30
20
10
0
SPF
P+O Vac.
O. Vac.
Natu. Infec.
Groups of chickens

FIG. 45:


Regression of passive IgG levels in the sera of chicks derived from parenterally -orally vaccinated chickens.
Against protein Ag
Against LPS Ag
Reciprocal dilutions of sera x 1000
40
30
20
10
0
1
5
10
15
20
25
30
Age (days)

(1982).

The egg shell was broken and the yolk was separated completely from the white. After cutting the yolk membrane, the yolk was poured into a glass beaker. Four volumes of Tris buffered saline (TBS) pH 7.3 were added to the yolk, mixed with a Sorvall Omnimixer Model 17106 (U.K.) for 1 minute at low speed and centrifuged (Beckman model J2-21 centrifuge) at 2000g for 20 minutes at 20°C and the pellet discarded. TBS was made by dissolving 10 mM of Tris (Hydroxy methyl) methylamine (BDH Chemicals Ltd.) in a solution of 0.15 M NaCl per litre with 0.1% Sodium azide as preservative (Johnstone and Thorpe, 1982). The pH was adjusted using HCl.

4 ml Dextran sulphate (10% w/v already prepared in TBS) and 10 ml of 1 M calcium chloride (in TBS) were added to the resultant 50-60 ml supernatant after separation from the pellet, mixed and left for 30 minutes at room temperature.

The mixture was centrifuged at 2000g for 20 minutes at 4°C and the supernatant was separated and stored. The pellet was resuspended in TBS to a final volume of mixing volume as in the first step (yolk+TBS), centrifuged and the 2 supernatants were pooled. IgG was precipitated using ammonium sulphate (Goudswaard et al. 1977a. Rose et al. 1974). First the solution was saturated up to 20%, centrifuged and the pellet discarded. Then ammonium sulphate was added to 40% saturation, centrifuged and the pellet dissolved in 5 ml of PBS and dialyzed against 3-4 litres of PBS for 24 hours by using a semipermeable dialysis bag (Medical International Ltd., London). Salt precipitation and dialysis were carried out at 4°C.

The dialysate was separated by chromatography on a Sephadex G-200 column (Pharmacia) 1 m long and 2.5 cm wide. Sephadex buffer [2.42g of Tris (Hydroxy methyl) methylamine/litre of deionised water with added 2% NaCl and 0.1% NaN_3. pH 8.] was used to elute the immunoglobulin. The

eluate was collected in 5 ml fractions, by using a spectrophotometer (Model SP6-550 UV/VIS, Pye Unicam Ltd., Cambridge, England). Their absorbance at 280 nm was measured and a curve plotted (Fig. 46). The fractions marked by the bar line were pooled and concentrated to the smallest possible volume in a semipermeable tube by using polyethylene glycol M.W. 2000 (BDH). The concentrated immunoglobulin solution was dialyzed for 48 hours against 2 changes of 2 litres of PBS buffer.

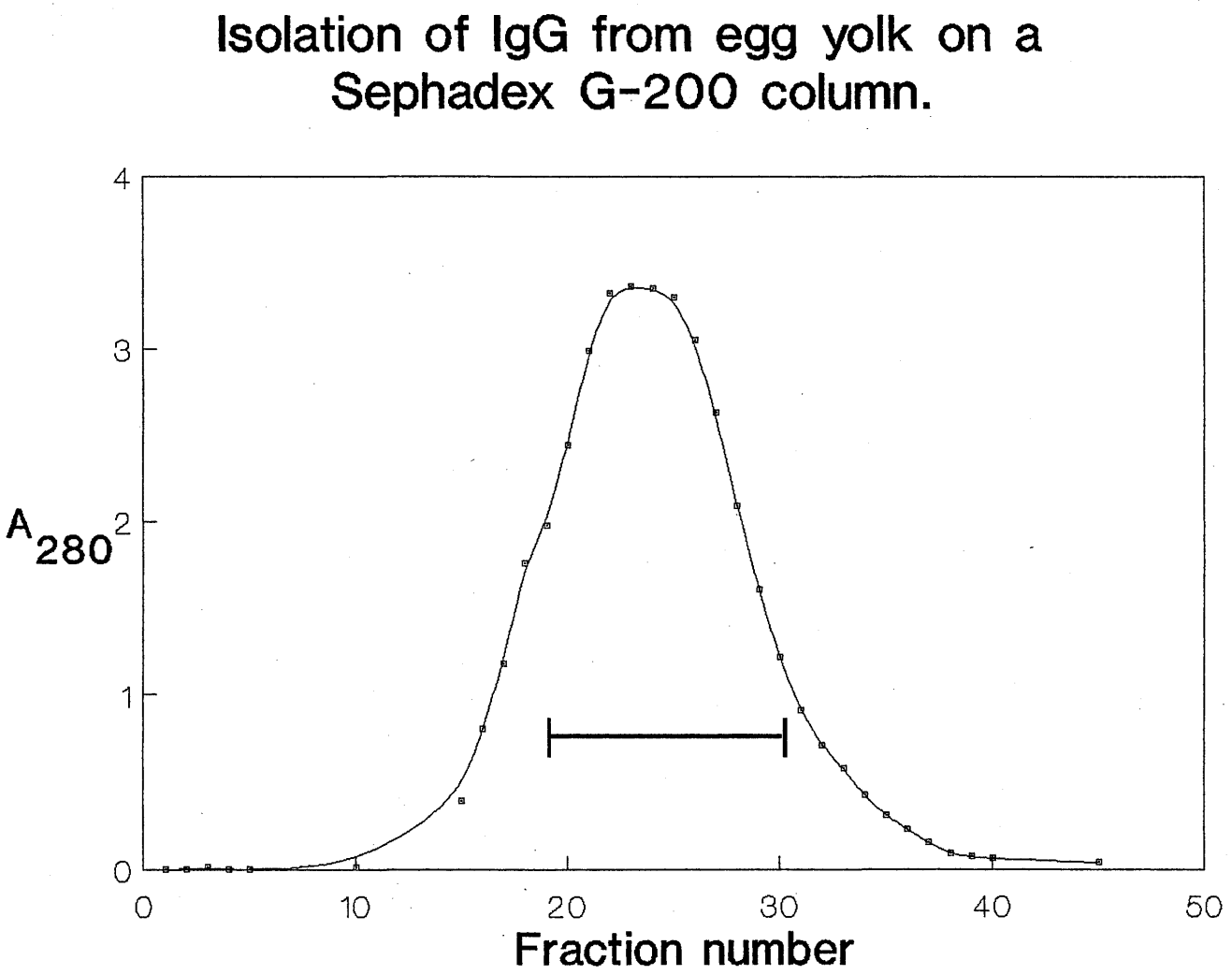
After dialysis, the solution was filtered first through a 0.45 um filter (Minisart type NML, Sartorius GmbH, West Germany) and then through a 0.22 um filter. The filtrate was collected in a sterile universal. The concentration of total soluble protein in this solution was found by Lowry's method to be 200 mg/ml and the presence of IgG was confirmed by using immunoelectrophoresis (Fig. 47 and 52) and the ELISA test (the end point dilution was 1:1,200,000 against both LPS and surface protein antigens). This solution was injected into SPF eggs as described below.

(ii) Isolation of IgA+IgM from egg white

The general concept for isolation of these 2 immunoglobulins was derived from the work of Rose et al. (1974). After breaking the egg shell, the egg white was carefully isolated from the yolk and pooled in a beaker. By using a Sorvall Omnimixer Model 17106, the pooled white was mixed at low speed for 1 minute. It was then acidified to pH 5.2 by adding an equal volume of 0.05M H_2SO_4 and centrifuged using Beckman Model J2-21 at 7000g for 20 minutes at 4°C. The pH of the supernatant was adjusted to 7 with 0.1M NaOH and ammonium sulphate was added to 30% saturation, centrifuged at 3000g for 20 minutes at 4°C and the pellet discarded.

The supernatant was saturated to 40% by adding further ammonium sulphate and then centrifuged at 7000g for 20 minutes

FIG: 46:


Isolation of IgG from egg yolk on a Sephadex G-200 column.
A280
0
1
2
3
4
0
10
20
30
40
50
Fraction number

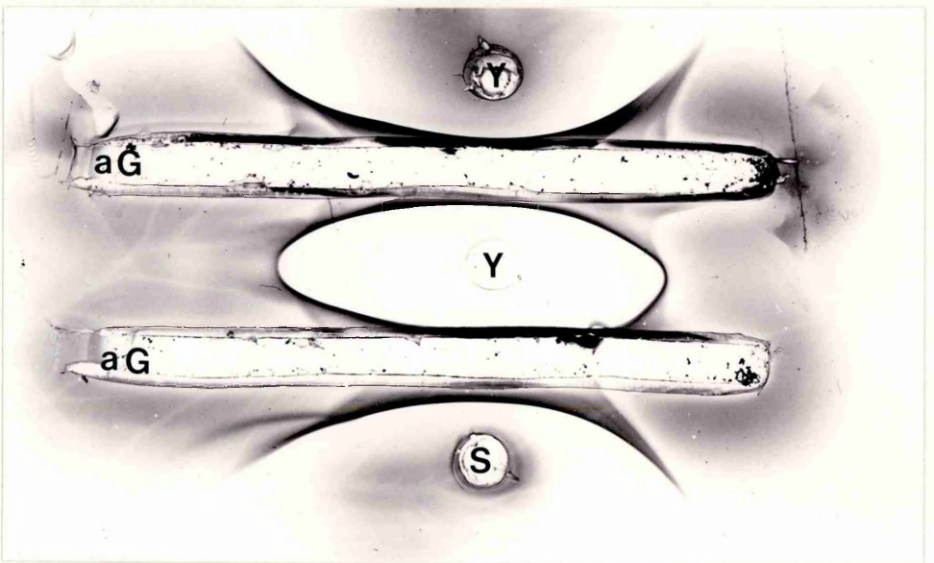


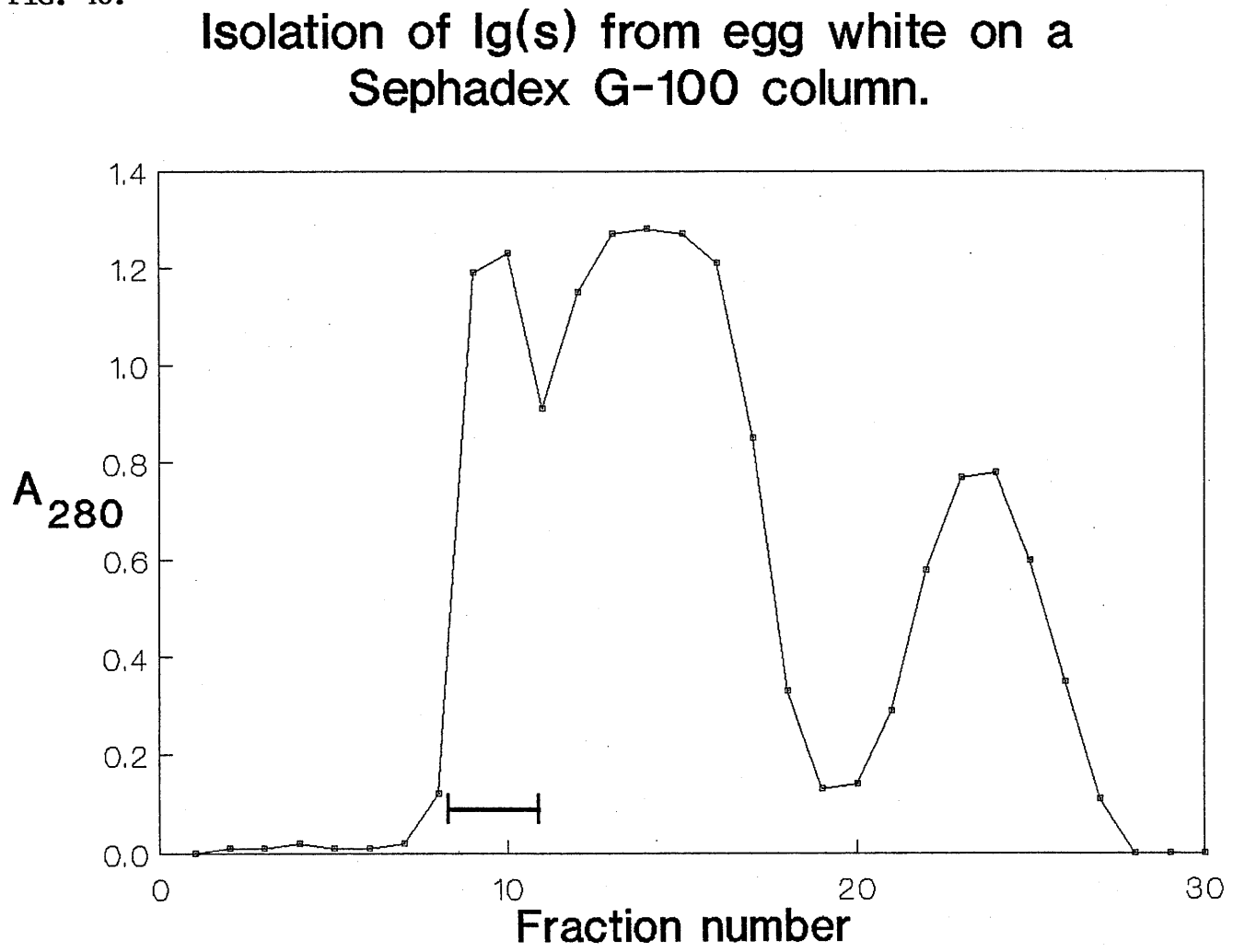
FIG. 47: Immunoelectrophoretic pattern of purified egg yolk IgG.

Y = Yolk IgG S = Hen serum aG = Anti-chicken IgG

at 4°C. The precipitate was dissolved in a small volume of distilled water and dialyzed against Sephadex buffer for 24 hours. The dialysate was concentrated using polyethylene glycol M.W. 2000 and dialyzed against Sephadex buffer for another 24 hours. It was then applied on a 16 mm wide, 70 cm long G-100 Sephadex column (Pharmacia). 5 ml fractions were collected and their absorbance was obtained at 280 nm (Fig. 48). The fractions under each peak were pooled separately and examined by double immunodiffusion and immunoelectrophoresis for the presence of IgA and IgM (Figs. 49 and 50). IgA and IgM were eluted together under the first peak. The fractions shown by a the bar line were pooled, concentrated dialyzed and applied on a G-200 Sephadex column (described above) for further purification.

The procedure described above, was repeated and the IgA and IgM that were eluted together under the main peak were collected (Fig. 51). The fractions showed by the bar line were pooled, concentrated and dialyzed against 2 changes of 2 litres of PBS buffer at 4°C. As filtration was not found to be a satisfactory method of sterilisation (the solution failed to pass through a 0.45 um filter), a mixed solution of penicillin+streptomycin (Cell culture reagent, Sigma) was added to the immunoglobulin solution immediately after dialyzing out the sodium azide and kept at 4°C for 24 hours before injection into the SPF eggs. The concentration of penicillin was 100 U and that of Streptomycin 0.5 mg/ml of the final solution. The concentration of protein in this solution was measured by Lowry's method (120 mg/ml) and the presence of IgA+IgM were confirmed by both immunoelectrophoresis (Fig. 52) and the ELISA test (the end point dilutions were 1:51200 for IgA and 1:25600 for IgM against both surface protein and LPS antigens).

FIG. 48:


Isolation of Ig(s) from egg white on a Sephadex G-100 column.
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A280
0
10
20
30
Fraction number

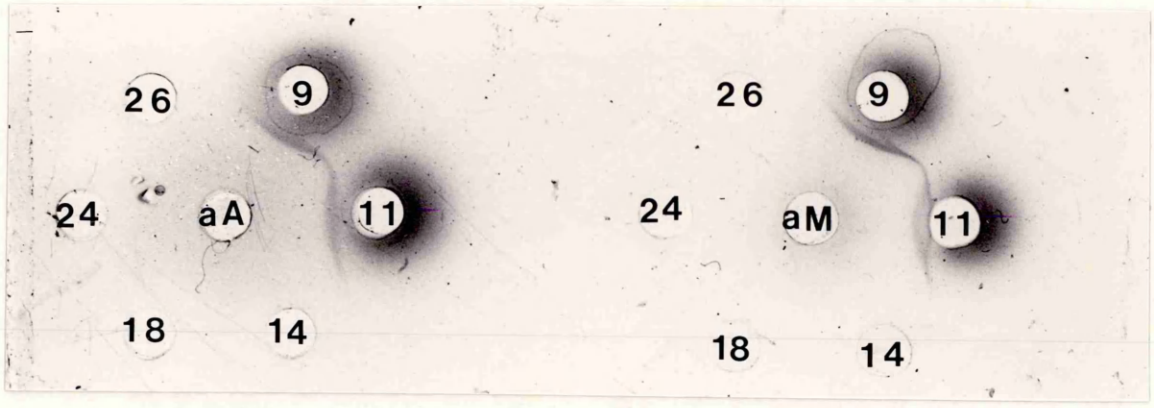


FIG. 49: Double immunodiffusion pattern obtained with eluate collected after application of egg white immunoglobulin fraction on Sephadex G-100 column.
Fractions 9 and 11 were collected under the first peak in Fig. 48 and the others were collected under peaks 2 and 3.
aA = Anti-chicken IgA aM = Anti-chicken IgM

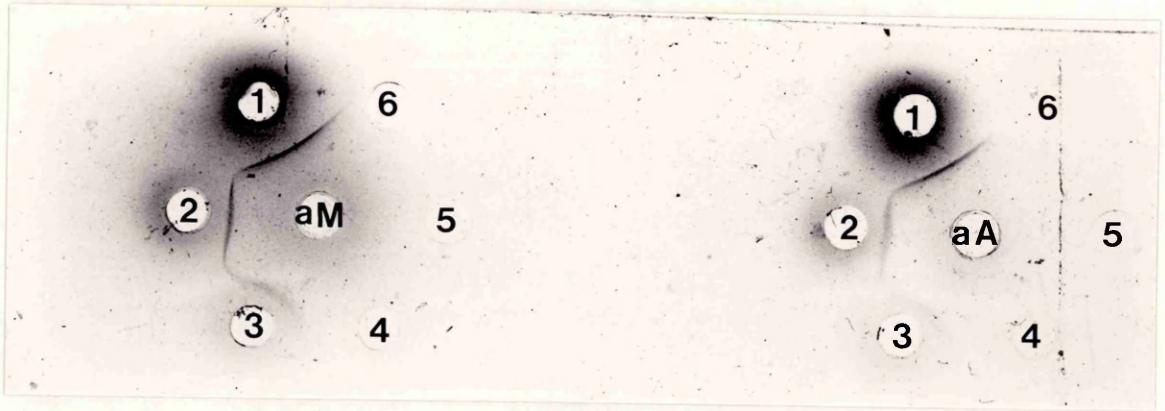
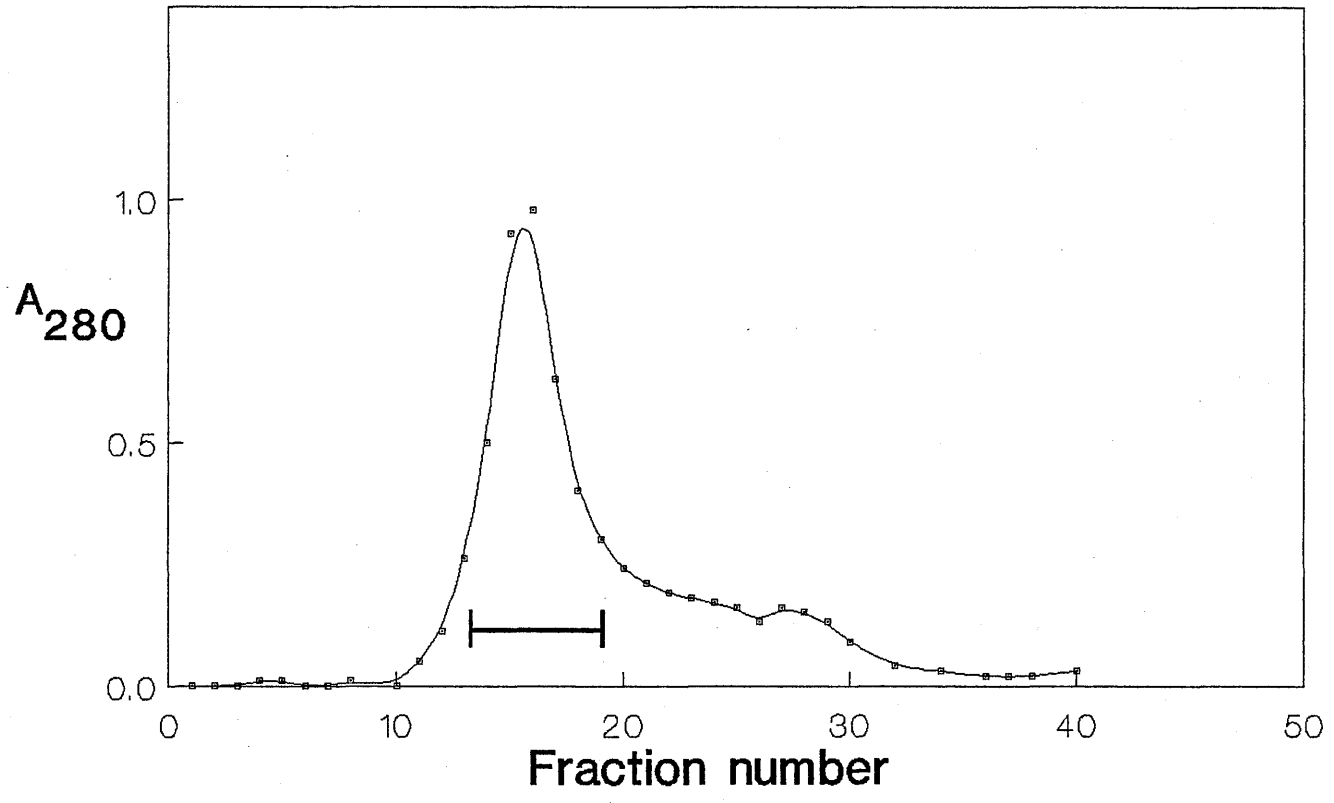


FIG. 50: Double immunodiffusion pattern obtained with concentrated pooled fractions collected under each peak of Fig. 48.
1 = Concentrated fractions of peak 1 2 = 1:10 dilution of 1
3 = Concentrated fractions of peak 2 4 = 1:10 dilution of 3
5 = Concentrated fractions of peak 3 6 = 1:10 dilution of 5
aA = Anti-chicken IgA aM = Anti-chicken IgM

FIG. 51:

Isolation of IgA+IgM from the pooled fractions of peak 1 in Fig. 48 on a Sephadex G-200 column.



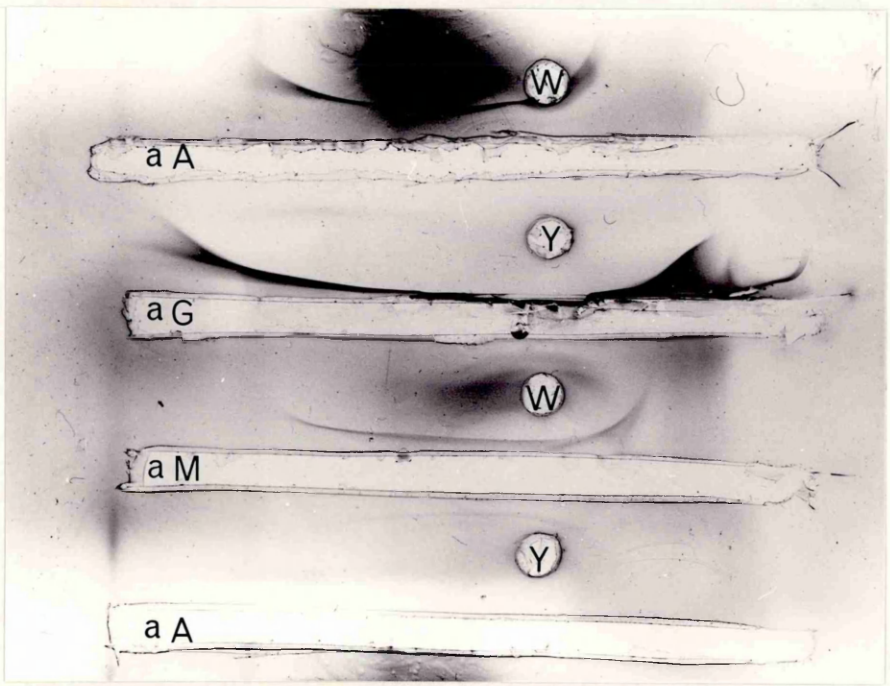


FIG. 52: Immunoelectrophoretic pattern of purified egg white IgA+IgM (W) and yolk IgG (Y).

aA = Anti-chicken IgA aM = Anti-chicken IgM

aG = Anti-chicken IgG

b) Injection of immunoglobulins into SPF eggs and challenge study

(i) Site and method of injection

The site for injection of IgG was the egg yolk and those for IgA+IgM were egg yolk, egg albumin and allantoic cavity. All injections were carried out at day 9-11 of incubation except injection into egg albumin which was done immediately prior to incubation. The injection methods used were those of Cunningham (1966).

Egg yolk: The eggs were taken from the incubator, candled and placed vertically on an egg tray with the air cell up. The area over the upper extremity (over the air cell) was disinfected with tincture of iodine. Using a sharp sterile needle, a small hole was drilled at the upper extremity of the shell over the air cell. A sterile 1 ml syringe and a 23 gauge, 33.3 mm (1.25 inch), sterile needle were used for injection. The needle was inserted to its full length vertically through the hole and 0.2 ml of the inoculum was deposited. The hole was sealed with melted paraffin wax and the egg returned to the incubator. At the end of the trial the incubator was fumigated with a light formaldehyde gas for 5 minutes. The injection techniques are shown in Figs. 53, 54 and 55.

Injection into allantoic cavity and egg albumin: The method was the same as that described above for injection into the yolk except that a 25 gauge, 16 mm sterile needle was used. Egg albumin injection was carried out just before incubating the eggs.

(ii) Challenge study

The following experiments were designed to test the effect of injecting immunoglobulins into different compartments of the eggs on the resistance of the chicks obtained to challenge.

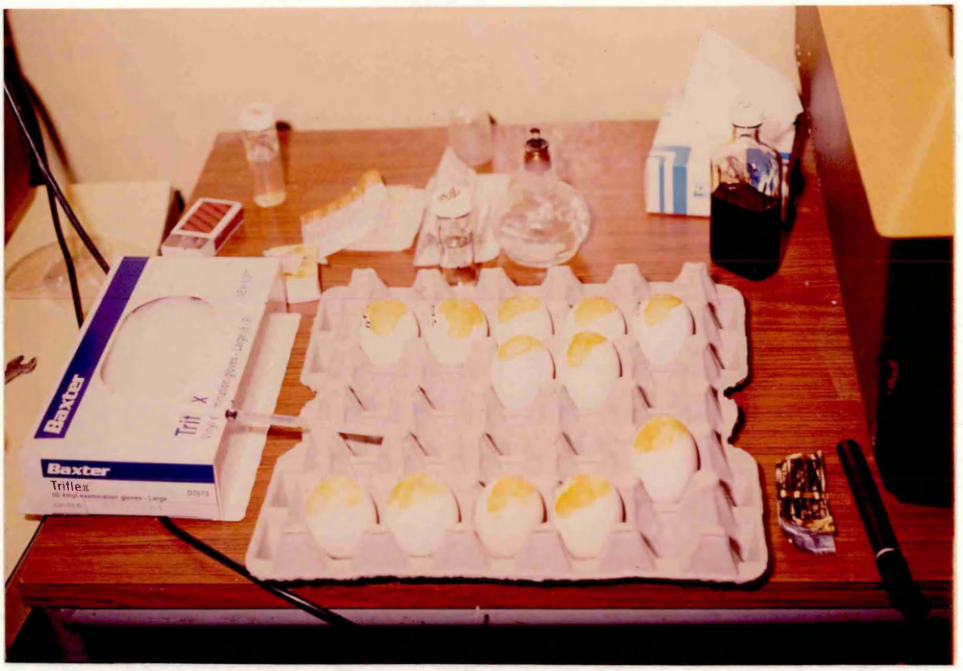


FIG. 53: Photograph of the equipment used for injection into eggs. The blunt end of the eggs was disinfected with tincture of iodine.

FIG. 54: Method used to make a hole at the blunt end of the egg over the air cell using a sharp sterile needle passed through cork.

FIG. 55: Injection into the yolk or allantoic cavity of embryonated eggs. The injected eggs are sealed using melted paraffin wax.

Experiment 8: In this experiment 3 groups each of 35 fertile SPF eggs were injected into the yolk sac. The eggs in group 1 were injected with 0.2 ml of IgG, group 2 with 0.2 ml of IgA+IgM and group 3 with 0.2 ml of sterile PBS (control group).

Experiment 9a: In this experiment 2 groups of 31 fertile SPF eggs were injected into the allantoic cavity. Group 1 eggs were injected with 0.2 ml of IgA+IgM and group 2 with 0.2 ml of sterile PBS. The hatch was very poor for reasons described below and this experiment was repeated as follows.

Experiment 9b: This was the same as Experiment 9a except that each group contained 16 fertile SPF eggs.

Experiment 10: In this experiment the egg albumin of 2 groups of 25 SPF eggs were inoculated prior to incubation. Group 1 eggs were injected with 0.3 ml of IgA+IgM and those of group 2 with 0.3 ml of sterile PBS.

The hatched chicks of each group were exposed to seeders that were orally inoculated with a fresh broth culture of S. typhimurium pt.49. In Experiments 8 and 9a the seeders were inoculated with $0.91x10^3$ CFU and in Experiment 9b with $0.75x10^3$ CFU of the organism. Methods of inoculation, accommodation and feeding were described in Chapter 2 Section 3.

In Experiment 8, 6 chicks from each of groups 1 and 3 and 5 chicks from group 2 were killed on day 7 post exposure and their organs were cultured. On day 11, 5 chicks from each of groups 1 and 3 and on day 14, 5 chicks from each of groups 1, 2 and 3 were killed and their hearts, livers and caeca were cultured. In Experiment 9a, 3 chicks from each group were killed on day 7 and the rest on day 14 post exposure. In Experiment 9b all chicks were killed on day 7 post exposure. As too few chicks had hatched in Experiment 10, the challenge study was not carried out.

c) Bacteriological monitoring

The immunoglobulin and the control solutions used as inoculum in this study were cultured on sheep blood, MacConkey and BG agar, incubated at 37°C, 29°C and 4°C aerobically and examined after 24 and 48 hours. They were also inoculated into tetrathionate broth and sub cultured onto BG, DCA and SS agar after 24 and 48 hours incubation at 37°C. These plates were incubated at 37°C and examined after 24 hours.

All of the unhatched eggs, embryos and dead chicks were examined for Salmonella infection.

The exposed birds were monitored by cloacal swabbing and killing at 7 days intervals and the organs cultured as described in Chapter 2.

B) Results

a) Hatching

The results of hatching are shown in Table 34. The best results were obtained in Experiment 8 (injection into yolk sac) in which 80% of the eggs injected with IgG (group 1), 65.7% of those injected with IgA+IgM and 74.2% of the control group (3) were hatched. In Experiments 9a and 10 the results of hatching were poor and in Experiment 9b were fair (68.75%). After some chicks died or were culled, 25 chicks were left in group 1, 17 in group 2 and 24 in group 3 of Experiment 8. Each one of these groups was exposed to 2 seeders. There were 7 chicks in each group of Experiment 9a. Both groups were kept together and were exposed to one seeder. In Experiment 9b there were also 7 chicks in each group and both groups were exposed to the same seeder. The hatched chicks from Experiment 10 were also kept in the same pen with these chicks.

Table 34

The hatching results of all groups of SPF eggs that were injected with immunoglobulins in Experiments 8, 9a, 9b and 10.

Experiment	Group	Result of hatch				
		Live chicks	Dead chicks	Total No.	Total %	Unhatched eggs
8	1	27	1	28	80.0	7
	2	20	3	23	65.7	12
	3	26	0	26	74.2	9
9a	1	7	1	8	25.8	23
	2	7	1	8	25.8	23
9b	1	11	0	11	68.7	5
	2	8	2	10	62.5	6
10	1	1	0	1	4	24
	2	8	2	10	40	15

b) Clinical observations

All unhatched eggs were visually examined for the presence or absence of embryos and estimation of their age. In Experiment 8, most of the unhatched eggs had a fully grown embryo which died at the last week of incubation and some were chipped but did not hatch. In Experiment 9a, group 1, out of 23 unhatched eggs, 18 contained a fully grown embryo which had died prior to absorption of the yolk and among them 5 were chipped. 3 had died around injection time and 2 at the end of week 2. In group 2 (Experiment 9a) 19 were fully grown, 3 of them had chipped and 4 showed absorption of yolk. The remaining four had died around week 2 of incubation. In Experiment 9b, there were 4 unhatched eggs in group 1 and 5 in group 2. All except one contained fully grown embryos that had died in the late stages of incubation.

In Experiment 10, group 1, 4 out of 24 unhatched eggs were infertile, 9 had died in the first week, 4 had grown to two weeks and 7 were fully grown and had died in the last stages of incubation. In group 2 of this experiment there were 5 infertile eggs, 7 of which had died in the first week, and 2 of which were fully grown and had chipped.

All hatched chicks were apparently healthy and normal prior to exposure to infection by seeders. The clinical signs of the disease produced were those described in the previous Chapters. No mortality was observed in group 1 of Experiment 8. In group 2, one chick was found dead outside the pen on day 9 post exposure, because it had jumped out and cut itself off from feed and water. At post-mortem examination showed slight congestion of the kidneys, urate deposits in the ureters and dehydration were seen. In group 3 one chick died on day 2 post exposure and signs of severe bleeding were found in the umblicus and Salmonella was isolated from the heart, liver and the caeca. No gross pathological lesions were observed apart from paleness of the carcass. Another chick from this group

died on day 11 post exposure. At post mortem examination fibropurulent pericarditis, perihepatitis and peritonitis with adhesion of visceral organs were found. The yolk sac was retained and filled with coagulated yolk. Salmonella was isolated from all of the organs that were cultured (Table 35).

In Experiment 9a, severe urate deposits on all visceral organs were seen in 2 chicks (one from each group). These chicks died on day 2 post exposure. Another chick died from group 1 of this experiment but the only gross lesion found was a slight urate deposit in the ureter. Salmonella infections in the chicks which died are shown in Table 35.

c) Bacterial isolation

No Salmonella or any other bacteria was isolated from the inocula under the conditions described above. All unhatched eggs were negative for Salmonella. A few (4 in total) unhatched eggs yielded streptococci on sheep blood and MacConkey agar but this finding was not limited to any specific group.

All cloacal swabs taken from the chicks prior to exposure were negative for Salmonella. The results of Salmonella isolation after exposure to seeders are described below.

Experiment 8: The results of cloacal swab culture are shown in Fig. 56. 75% of chicks in group 3 (control) were infected on day 1 post exposure, but only 11.76% in group 2 and 12% in group 1 were infected. On day 2 post exposure, 60% in group 1, 52.9% in group 2 and 100% in group 3 were infected. On day 3, 96% in group 1 and 94.1% in group 2 were infected. The difference among the groups had disappeared by day 4, when they were showing 100% infection (Table 36 and Fig. 56).

The results of Salmonella isolation from internal organs are shown in Table 37. On day 7 post exposure the caeca of all chicks were colonised. Systemic infection was observed in one

Table 35

Mortalities and the status of *Salmonella* infection in the experimental groups used in the challenge study with *S. typhimurium* pt. 49 in Experiments 8, 9a and 9b.

Experiment	Group	No. of chicks died	Organs cultured	*Salmonella* isolation	
				Direct	Indirect
8	1	0	---	---	---
	2	1	Heart	0:1	0:1
			Liver	1:1	1:1
			Caecum	1:1	1:1
	3	2	Heart	2:2	2:2
			Liver	2:2	2:2
			Caecum	2:2	2:2
9a	1	1	Heart	1:1	1:1
			Liver	1:1	1:1
			Caecum	1:1	1:1
	2	3	Heart	0:3	2:3
			Liver	0:3	2:3
			Caecum	3:3	3:3
9b	1	2	Heart	0:2	0:2
			Liver	0:2	0:2
			Yolk sac	0:2	0:2
			Caecum	1:2	1:2
	2	1	Heart	0:1	0:1
			Liver	1:1	1:1
			Yolk sac	1:1	1:1
			Caecum	1:1	1:1

FIG. 56:

Faecal carriage of S. typhimurium in chicks from eggs injected with immunoglobulins in Experiment 8.

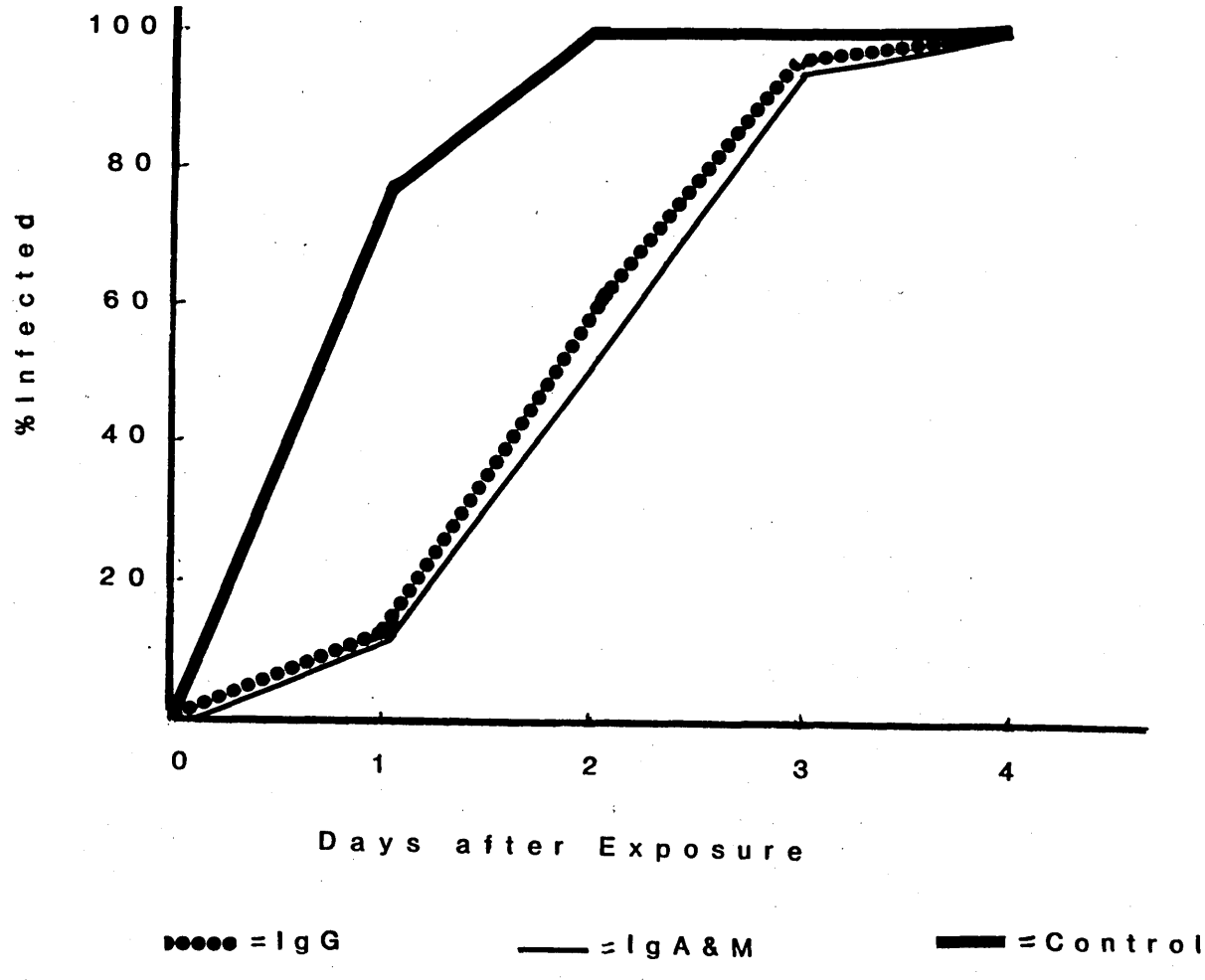


Table 36

Results of cloacal swab culture for Salmonella isolation from chicks in all groups of Experiments 8, 9a and 9b.

Experiment	Day after exposure	Group 1		Group 2		Group 3	
		No.*	%**	No.	%	No.	%
8	0	0:25	0.0	0:17	0.0	0:24	0.0
	1	3:25	12.0	2:17	11.8	18:24	75.0
	2	15:25	60.0	9:17	52.9	24:24	100
	3	24:25	96.0	16:17	94.1	23:23	100
	4	25:25	100	17:17	100	23:23	100
9a	0	0:7	0.0	0.7	0.0	---	---
	1	3:7	42.9	4:7	57.1	---	---
	2	7:7	100	7:7	100	---	---
	3	7:7	100	7:7	100	---	---
	4	7:7	100	6:6	100	---	---
9b	0	0:7	0.0	0:7	0.0	---	---
	1	1:7	14.3	2:7	28.6	---	---
	2	5:6	83.3	7:7	100	---	---
	3	5:5	100	6:6	100	---	---
	4	5:5	100	6:6	100	---	---

* = No. of positives:Total No. of birds.

** = Percent infected.

Table 37

Salmonella isolation from the organs of chicks killed at different time intervals in Experiments 8, 9a and 9b.

Exp.*	Days after exposure	Organs cultured	Group 1		Group 2		Group 3	
			Direct	Indir.	Direct	Indir.	Direct	Indir.
8	7	Heart	0:6	0:6	1:5	2:5	0:6	0:6
		Liver	0:6	1:6	2:5	4:5	1:6	1:6
		Caecum	0:6	6:6	2:5	5:5	0:6	6:6
	11	Heart	0:5	0:5	---	---	0:5	0:5
		Liver	0:5	0:5	---	---	0:5	0:5
		Caecum	1:5	5:5	---	---	2:5	5:5
	14	Heart	0:5	0:5	1:5	1:5	0:5	0:5
		Liver	0:5	0:5	1:5	1:5	0:5	0:5
		Caecum	1:5	5:5	1:5	5:5	1:5	5:5
9a	7	Heart	0:3	0:3	0:3	0:3	---	---
		Liver	1:3	3:3	1:3	3:3	---	---
		Caecum	2:3	3:3	1:3	3:3	---	---
	14	Heart	0:3	0:3	0:1	0:1	---	---
		Liver	0:3	1:3	0:1	1:1	---	---
		Caecum	1:3	3:3	0:1	1:1	---	---
9b	7	Heart	0:5	0:5	0:6	0:6	---	---
		Liver	0:5	0:5	1:6	2:6	---	---
		Caecum	4:5	5:5	3:6	6:6	---	---

chick of each of groups 1 and 2 and in four chicks in group 3. On days 11 and 14 the presence of Salmonella was confirmed in the caeca of all chicks killed and one of the chicks in group 2 (day 14) showed systemic infection.

Experiments 9a and 9b: The results of cloacal swab culture are shown in Table 36 and those of the internal organ culture in Table 37. In both experiments the chicks started shedding Salmonella on day 1 post exposure and nearly all of them were colonised by day 2 and there was no marked difference between chicks of immunoglobulin injected eggs and control chicks in either experiment. Caecal colonisation was demonstrated in all chicks in these experiments by day 7 and systemic infection was observed in all groups except group 1 in Experiment 9b.

4 - DISCUSSION

The main aims of this study were:

a) To determine the titres and persistence of the specific immunoglobulins to S. typhimurium pt. 49 antigens in the sera of young chicks which had been derived from the eggs of immunised hens.

b) To study the role of each of these immunoglobulins in the protection of young chicks when challenged with the same organism.

A) Immunoglobulins in the chick sera

The eggs used in this study were laid by the parenterally-orally vaccinated hens and had been shown to have high titres of antibodies to surface protein and LPS antigens of S. typhimurium pt. 49 (Chapter 4). Salmonella was not isolated from the unhatched eggs and chicks during the study. The results presented show high levels of all 3 classes of

immunoglobulin in the sera of these chicks when compared to the sera taken from chicks derived from SPF, orally vaccinated and naturally infected hens. The presence of specific antibodies was demonstrable by both ELISA and agglutination tests until 15 days of age. From this time on, only the ELISA was capable of detecting these antibodies.

The highest titres for all 3 classes of antibody were observed in the sera of newly hatched chicks and remained high until day 5 and then decreased with age. The highest titres were observed for IgG against both antigens , with those against surface protein reaching 1:38400 (Table 33) compared with 1:9600 for LPS (Table 33). These titres fell by half every 5 days and this decrease was observed daily until day 30 of age.

Due to the difficulty of bleeding of young chicks from the wing vein, cardiac puncture had to be used and such chicks were killed. Consequently the sera obtained at each time interval were from chicks other than those bled previously. For this reason the fall in antibody levels could not be followed in individual chicks during this experiment. In the other groups of chicks examined, IgG antibody to surface protein antigen was only higher in 2 chicks from naturally infected hens than that in SPF chicks.

The specific IgA and IgM antibody was only detected in serum of chicks derived from parenterally-orally vaccinated hens. These immunoglobulins were detected on an ELISA plate also being used for the sera of chicks from naturally infected, orally vaccinated and SPF hens. No specific IgA or IgM antibody was detected in the other sera. The use of the same plate and of these control sera suggests the result was genuine. The same result was obtained when the exercise was repeated. Therefore it is possible to conclude that these immunoglobulins present in the sera of these young chicks were derived from the immunised hens. The presence of these 2 immunoglobulins in the

eggs of the parenterally-orally vaccinated hens was described in Chapter 4. The levels for all 3 classes of immunoglobulins in the sera of newly hatched chicks were higher than of those found by bulk examination of the egg yolk and egg whites described in Chapter 4. This might be due to variation among individuals or to concentration of these immunoglobulins when transferred to sera. Concentration is possible as volumes of both yolk (14-18 ml) and white (25-32 ml) are much higher than that of the total blood volume of chicks (3-5 ml). The selective transport of IgG from yolk to the serum of the chick has been reported by Tizard (1982). The mechanism of this transfer is described by Kowalczyk et al. (1985), but as yet the presence of IgA and IgM in serum of newly hatched chicks has not been reported, the mechanism involved in its transfer must be a matter of further investigation. Possible ways in which transfer could occur are that:

a) IgA and IgM move into amniotic fluid during embryonic growth and from there are swallowed by the chick during the last stages of incubation (Tizard, 1982). They may then be absorbed from the gut into the circulation.

b) Through the embryonic circulation as for IgG.

The presence of specific IgG to some bacterial and viral pathogens in the serum of newly hatched chicks is reviewed in Chapter 1. These reference accounts are borne out by the finding of specific IgG against both antigens of S. typhimurium pt. 49 in chick serum in this study.

B) Studies on artificial transfer of the immunoglobulins to eggs

As described in Chapter 1, newly hatched chicks are immuno-incompetent, therefore the partial resistance found against Salmonella infection in chicks derived from naturally infected or vaccinated hens is probably due to the presence of

specific antibodies in the eggs. The presence and the transfer of these antibodies is described above. In this study it was considered necessary to prove this supposition by artificially transferring of one or more of these immunoglobulins to eggs that were free from them.

IgG, IgA and IgM were purified from the eggs of parenterally-orally vaccinated hens. The levels of the specific antibodies in these eggs has already been described and was further examined after purification by the ELISA test. High titres (1:1,200,000 for IgG, 1:51,200 for IgA and 1:25,600 for IgM) indicated not only the presence of these antibodies in the purified extract but also shows that the antigen binding sites were not altered during the process of purification. The presence of these immunoglobulins was also confirmed by immunoelectrophoresis (Figs. 47 and 52). IgA and IgM were eluted together in both G-100 and G-200 Sephadex columns, probably because of their close molecular weight and were used as a mixture.

In theory the sterile conditions and the filter size (0.22 um) used for the filtration of IgG should have excluded pathogenic organisms from that solution. No bacteria were isolated from the filtrate under the aerobic conditions described. IgA and IgM did not pass through filters suitable for the exclusion of bacterial and viral pathogens. Their sterility relied on the hygienic conditions employed and addition of penicillin and streptomycin. These antibiotics were also added to the PBS solution used for the injection of control eggs for this group. Culture for bacteria did not reveal any growth from this solution and the evidence presented from hatching results and visual and bacteriological examination of the unhatched eggs did not indicate the presence of pathogens.

Failure of the eggs injected with IgA+IgM (Experiment 9a) to hatch was due to an incubator fault leading to a lower

temperature than that required. The results of the protection study suggested that the eggs injected with IgG had higher hatchability (80%) than those injected with PBS (74.2%) or IgA+IgM (65.7%) in the same site (Table 34). IgA+IgM injection into the allantoic cavity showed (68.7%) hatch and that in control group was 62.5%. Injection into the yolk sac had little effect on hatchability although a variation was observed among different groups (injected with different inoculum) in contrast to injection into the egg albumin which dramatically reduced the hatchability.

The eggs received from this SPF flock and hatched without injection for challenge studies during this study had hatching percentages of 35.71% to 95% with an average of about 80%. Comparing with controls and a normal hatch the inocula did not decrease the hatchability although the effect of injection stress on embryos and hatchability must be further investigated. Controls with untreated eggs were not used, so, the exact hatch rate for eggs used in the injection studies is not known. The recommended dosage for injection into allantoic cavity is 0.1-0.2 ml and that for yolk sac injection is 0.2-1 ml (Cunningham 1966). Therefore the dosages used in this study were within these limits and should not have decreased the hatchability. In Experiment 10 the IgA+IgM was injected into albumin of the SPF eggs. Controls received PBS only. Both inocula contained antibiotics as described. But the result of hatch was disappointing. From the evidence obtained from bacterial culture and examination of unhatched embryo, bacterial contamination can be ruled out and no signs of viral infection were present. As embryos did not die at one stage several factors could be blamed for this fault. The mortality was comprised as follows: a) 16-20% infertile eggs (clear when examined) b) 30-40% of embryos died in the first week possibly due to the stress of injection. c) The rest died in the late stages of incubation may have had a problem such as coming from a senile parent, from parents with low quality of nutrition or from those under stress etc. The reasons for this failure can

only be speculation as no published information about this procedure exists.

In the challenge study the best results were obtained with IgG injection into the yolk and IgA+IgM in the same site. Colonisation was delayed by up to day 4 post exposure (Table 36 and Fig. 56) in the chicks that received the immunoglobulins but on day 4 this difference disappeared.

Mortality was completely prevented in all 25 chicks by IgG injection into the yolk, but in both group 2 and 3 mortality occurred (Table 35). The results were less clear when the culture results from day 7 were considered as Salmonella was isolated from the liver of one chick from group 1 after enrichment. On days 11 and 14 post exposure systemic infection was observed in group 2 only. Blood samples from the artificially protected chicks were not examined for antibody and the presence of bacteria in the liver and heart may reflect differences in the levels of antibody transferred for technical reasons. The results clearly show that IgG or IgA+IgM when injected into the yolk were able to delay gut colonisation and there was a marked difference (Fig. 56) between these two groups and those that were injected with PBS. In Experiment 9b a marginal difference was found in both gut colonisation (Table 36) and systemic infection (Table 37) in chicks that had received IgA+IgM injection into allantoic cavity when compared with their control group. In Experiment 9a no difference was found between experimental and control groups. The results presented do not support a role for IgA+IgM when injected into the allantoic cavity. This study was not designed to show the mechanisms of action of immunoglobulins in newly hatched chicks when exposed to infection. However, the findings provide a reason for further investigation of the role of humoral immunity in resistance to Salmonella infection. At this stage it is not possible to underline the exact mechanism of action of these passively transferred antibodies. It may be to attach to the antigenic sites of the organism and render it inactive

and so render it unable to colonise in the gut. The fact that constant exposure of the chicks to infection changed the situation in favour of the organism might support this view. The immunoglobulins decline with the age of chicks while the organisms are still there. As the chicks are not equipped with an efficient immune system at this age, active immunity can play little part but a synergistic effect between passive immunoglobulins and body defence systems cannot be ruled out. Akeda et al. (1981) reported that macrophages appeared to supplement antibody in protection against S. typhimurium infection in mice.

CHAPTER 6

GENERAL DISCUSSION

1 - INTRODUCTION

In this chapter the ELISA and the results of Chapters 3, 4 and 5 are discussed in term of their importance and application in the diagnosis and prevention of poultry salmonellosis and their contribution to knowledge of the immunophysiology of the chicken. The results of the individual studies and their validity have already been discussed at the end of each chapter.

The order of discussion is firstly the ELISA, and then the results of colonisation studies and infection in young chicks. The role of vaccination and natural infection in the stimulation of antibody production is discussed next followed by the protection afforded in each study and the role of antibodies in the protection of day old chicks against infection. All are discussed in the context of the existing literature.

The discussion ends with a brief section of conclusions.

2 - THE ELISA

It is less than two decades since the enzyme immunoassay test was introduced by Engvall and Perlmann in 1971 (Ashorn and Krohn 1986). It was an alternative to the radio immunoassay and was readily accepted by investigators assaying biological materials. It was highly sensitive, easy to perform, and did not use the radioactive materials and expensive equipment necessary to measure the results of RIA. These factors made the ELISA acceptable to research workers (Kemeny and Challacombe 1986) and it is now used in both immunological research and routine tests in most aspects of diagnosis in biological

sciences.

The ELISA developed in this study has not been described previously, but follows the general concepts of similar ELISAs used on other animal samples. It was modified to make it the test of choice for the purposes of this study. The specificity and sensitivity of the ELISA were described in Chapter 2. Cross reaction has been reported between the H antigens of different Salmonellae (Bettelheim and Maskill 1985) and also between the LPS of Salmonellae and other members of the Enterobacteriaceae (Barclay and Scott 1987). Sanitation, isolation, providing of irradiated feed and cooled boiled water and controls were among the measures that were used to exclude multiple infection in this study. The feed used was pelleted prior to irradiation. Pelleting itself destroys Salmonella contamination (Zander 1984), further reducing the possibility of Salmonella antigen from this source. The results discussed above in Chapters 3 and 4 confirm there was no infection with other pathogenic Salmonellae and therefore the results obtained were the effect of the strain of Salmonella used in this study.

The light chains of chicken immunoglobulins can cross react with each other (Higgins 1975). For this reason Fc specific goat anti-chicken was used in the ELISA to avoid this problem. No cross reaction has been reported between the H chains of the chicken immunoglobulins. All three classes of specific immunoglobulins are capable of binding to the antigen on the solid phase, because the Fab of IgA, IgM and IgG may bind to the same antigenic determinant (Glick 1986). Therefore, they could compete with each other for binding sites and cause underestimation of the levels of the others. To increase the sensitivity of the test it is possible to block the activity of the immunoglobulins which are not to be tested. In this study the competing effect was not observed and the removal of IgG from samples did not increase the levels of these immunoglobulins, possibly because a titration was used in which the end point was determined by serial dilution.

Using negative standard serum for IgA and IgM studies, slightly high absorbances were noted. When these sera were treated with antigens as described in Chapter 2 and used again, lower absorbance was observed (Figs. VII-X, Appendix 1). This indicates either that the SPF sera had some degree of specific activity as a result of the exposure of hens to antigens or that there is a degree of non specific reaction between antigens and the serum. The cut off point of 0.1 used was at least twice the highest absorbance value obtained from the negative serum and eliminates the problem.

Standards and controls were also included on every plate to monitor any variation of the test in day to day experiments.

The ELISA constructed here was capable of detecting specific immunoglobulins in all the sites sampled in infected chickens and could be used for monitoring commercial flocks for Salmonella infection by replacing the common antigens of Salmonella with specific antigens of the expected strain. It can demonstrate the presence of specific IgG in the yolk and layer flocks could therefore be tested without the necessity of bleeding and at a low cost by using rejected eggs e.g. broken, cracked, shell less etc. Serum, bile, secretions and other fluid samples can be used as well.

There was a correlation between the ELISA and agglutination test used on serum samples. The higher sensitivity of the ELISA test could replace the current agglutination test for pullorum disease. Monoclonal antibodies can substitute for the polyclonal antibodies used in this test for higher specificity. Mockett et al. (1987) used an ELISA to measure maternally derived specific IgG against infectious bronchitis in the egg yolk and tracheal washings of one day old chicks. Nardiello et al. (1985) used LPS antigen of S. typhi to measure specific IgM against this antigen in the sera of patients with typhoid fever. Voller et al. (1979) proposed the ELISA for the diagnosis of all bacterial, viral, mycotic and

parasitic diseases.

3 - EXPERIMENTAL STUDIES WITH S. TYPHIMURIUM PT. 49

A) Salmonella infection

Oral inoculation with low doses (0.2 x 10^3 CFU) of S. typhimurium pt. 49 resulted in the colonisation of the intestine of day old SPF chicks, supporting the findings of Seuna (1979) who showed that a dose of 15 S. typhimurium bacteria was able to produce infection in day old chicks. Oral infection of chickens with S. typhimurium has also been reported by Barrow et al. (1987a), Williams (1984) and Brown et al. (1975). Following the infection Salmonella was isolated from most parts of the alimentary tract with highest numbers from the caecum in which the organism persists longest. Similar findings have been reported by Barrow et al. (1987a), Brown et al. (1975) and Sadler et al. (1969). The level of Salmonella shedding declined with the age of the chicks, but they remained carriers for up to 6 months of age. Sadler et al. (1969) showed that a large number of birds inoculated with S. typhimurium contained the organism in the lumen of their intestine 115 days later. The results presented here clearly indicate that this strain of Salmonella was able to colonise the intestines of the chicken. The mechanism of colonisation is not known but the findings of this study do not support the view that direct attachment (adhesion) of Salmonella to the caecal mucosa occurs (Soerjadi et al. 1982). Adhesion of Salmonella like bacteria to the brush borders of epithelial cells was demonstrated in the small intestine but did not appear to be a feature of caecal colonisation. Barrow et al. (1988) reported that the 85 Kilo base virulence associated plasmid, flagellar antigens, somatic antigens and mannose-sensitive haemagglutinins are not essential for colonisation of the chicken gut by S. typhimurium and S. infantis. They also suggested that non specific factors such as the slow rate of flow of the contents or host specific factors might be responsible for the persistence and

localisation of the Salmonella in the caeca.

In this study Salmonella was isolated from the gut of all infected chicks, but some of them yielded the organism from their heart, liver, spleen, etc. The latter indicates that some infected chicks may develop systemic infection. Therefore, for diagnosis of the infected birds bacteriological tests such as cloacal swabbing, faecal or litter culture (Brown et al. 1975) should be supplemented by serum antibody tests. Some infected birds may stop shedding the organism after a period of excretion. Thus, negative results of cloacal swabbing or faecal culture alone are not enough to confirm that flock is free from infection. It should be stated here that the decline of Salmonella shedding begins only 2-3 weeks after the start of infection (Brown et al. 1975) and cloacal swabbing such as that used in this study was carried out at the early active stage of infection which was in accord with the isolation of Salmonella and its recovery from the intestine (Brown et al. 1975, Sadler et al. 1969). The clinical signs, gross lesions and histopathological findings are all in agreement with those described by Williams (1984).

B) Antibody studies

Specific antibodies to surface protein and LPS antigens of S. typhimurium pt. 49 were found in serum, eggs, bile and oviductal washings of naturally infected chickens, parenterally-orally vaccinated hens and sera of the newly hatched chicks derived from eggs of these vaccinated hens. All 3 major immunoglobulin classes were found in the sera of the chickens in these 3 groups. IgG was the major immunoglobulin in serum (Tizard 1982) followed by IgM and then IgA (Chhabra and Goel 1980). These 3 immunoglobulins are an important part of the humoral defence mechanism. Their levels vary in the individuals. Not all of the naturally infected chickens had high levels of the specific immunoglobulins probably due to the reasons described above and in Chapter 3, Section 4. The

response of vaccinated chickens for antibody production was more uniform. IgA and IgM antibody was found in bile, oviductal secretions and in egg white of these chickens. The presence of specific IgA and IgM to S. enteritidis is reported by Jackson and Walker (1983) in rat bile. This is the first report of the presence of specific IgA and IgM to bacterial antigens in the oviductal secretions and egg white. Ewert et al. (1979) reported the presence of specific IgA and IgM to Newcastle disease virus in the egg white. As these antibodies were also found in these sites in the orally vaccinated hens, it suggests that these immunoglobulins are produced locally and they do not seem to have a direct relation with those in blood circulation. The work of Dohms et al. (1978) already cited in the previous chapters supports this view. On the other hand, IgA levels in the egg white of the parenterally-orally vaccinated hens increased after vaccine boosters were given which means that both oral and parenteral vaccines were able to stimulate secretory IgA production while only parenteral vaccination was able to stimulate its production in the circulation. Surprisingly these effects were only observed in the vaccinated hens with the most obvious effect in parenterally-orally vaccinated hens and to a lesser extent in orally vaccinated chickens. In naturally infected groups some of the hens (Tables 11, 13 and 17, Figs. 26 and 27) had equal or lower levels of IgA and IgM to compared with those of the SPF group which may reflect the early clearance of the organism from their gut which in turn indicates that the presence of antigen is necessary in the gut or circulation to stimulate local antibody production in the oviduct. If we consider that the naturally infected chickens were in an isolation unit but not in an SPF environment, while the orally vaccinated hens were in SPF conditions up to 16 weeks of age and then kept in an isolation unit more restricted than that of naturally infected hens, it is possible to conclude that this effect is mainly due to vaccine and other probabilities can be rejected on this basis. The naturally infected hens were colonised in the early part of their life and the differences in the antibody levels in their

oviducts can be explained by the hypothesis that exposure to antigen stimulates immunity only at the time of sexual maturity. At this time the oviduct grows rapidly in size and antibody secreting cells may migrate to it. In these groups of hens, levels of antigen may not have been enough to stimulate the relocation of plasma cells in some birds. Others that were still gut carriers or even had systemic infection might have developed higher populations of plasma cells producing specific antibodies against the organism in this site.

IgG was found to be the only immunoglobulin in the yolk which is in agreement with the findings of Rose et al. (1974) and Kramer and Cho (1970). The presence of high levels of specific IgG in the yolk is very interesting, because it can be used for detection of infection in laying flocks. It may not reveal the current situation of infection, but has the advantages described below:

a) Excludes the stress of bleeding .

b) More frequent sampling is possible.

c) Untrained people can be used for collection of samples.

The presence of maternally derived IgG in the sera of young chicks has been reported by Rose et al. (1974) and Kowalczyk et al. (1985), but the presence of IgA and IgM in chick sera has not been reported previously. Further to this, Rose et al. (1974) reported that there is no IgA or IgM in the sera of newly hatched chicks. Since this is the first type of experiment of its kind on the sera of newly hatched chicks, the result may reflect the difference in sensitivity of the ELISA test used here in comparison to immunoelectrophoresis used by these authors. It is interesting to know that chicks of this age cannot produce these immunoglobulins (as described in Chapter 1) and according to Toivanen et al. (1981), exposure to antigenic stimuli or keeping in germ free conditions during

incubation or after hatch does not affect the function and morphology of the bursa of Fabricius. Rose and Orlans (1981) reported that IgM production in the embryo does not respond to antigen injection. So, the IgA and IgM found in the chick sera may be derived from the humoral antibody response of the dam which is transferred via the egg white to the chicks.

The presence of IgA and IgM in bile has already been discussed in Chapter 3, Section 4.

C) Vaccines

In general live vaccines could be produced and applied easily and they can produce a uniform result in the flock (specially the viral vaccines). Killed vaccines do not spread the infection but they may cause less systemic reaction which is depending on the route of application. Not all infectious agents retain their immunising ability when killed.

Among the Salmonella vaccines which have been used in the past, both types have been shown to be effective. Live vaccines were usually resulted in the isolation of vaccinal strains from the chicken and killed vaccines were affected by method of preparation and routes of application. Never the less, there is no firm agreement among investigators that either type of vaccine is superior. Killed Salmonella vaccine was chosen in this study because of crucial importance of S. typhimurium to public health especially in laying flocks, where they can pass the organism to the public via eggs or broiler meat. Live vaccines may produce some degree of immunity without being pathogenic for poultry but their persistence in gut contents and carcasses may complicate bacteriological monitoring for public health purposes.

The evidence presented clearly indicates that the humoral immune system of the chicken is activated by both live bacteria and killed Salmonella vaccine and that its response is the same

as to other antigens. Natural infection did not produce a uniform response in all chickens. It is possible that those with no antibody were only colonised in the gut. Therefore, it is possible that live vaccines may not be much better than killed vaccine when introduced orally as killed oral vaccine failed to produce systemic reaction and live bacteria did not produce a systemic response in every bird. Suphabphant et al. (1983) reported that the live oral vaccine G30D does not produce a systemic response in every bird. The parenteral vaccine was used as recommended by Thain et al. (1984). Both primary and secondary responses were observed after the first and second injections of this vaccine (Glick 1986) and an anamnestic effect was also noted (Figs. 35-41). In some birds the antibody titre after first vaccination was the same as that after the second injection (Appendix 4) which may reflect the effects of the adjuvant used with vaccine. The dosage of oral vaccine follows the original proposal of Truscott (1981) who showed it to be effective against direct challenge in broiler chickens. Enrichment of the vaccine with surface protein antigen was carried out because of the importance of protein antigens in the protection induced by Angerman and Eisentein (1980) and emphasised by Barber and Eylan (1976). The protective effect of flagellar antigens in other bacterial species such as Clostridium chauvoei was described by Chandler and Gulasekharam (1974). As there are shared antigens on the flagella of some motile Salmonella (McDonough 1965), the inclusion of protein antigen could provide additional cross protection after vaccination with this antigen. Cross protection by antibodies to proteins of Salmonella against other Enterobacteriaceae was shown by Barber and Eylan (1976). Many types of live and inactivated vaccines have been developed and used in the past as described in Chapter 1. None of them could produce solid protection against subsequent infection but partial protection is described. The vaccine used in this study was able to produce levels of systemic antibody in the dam which led to prevention of systemic infection and delay in gut colonisation in newly hatched chicks. Lack of knowledge about

the mechanism of this protection, confusion and controversy over the type of immunity involved, poor understanding of the mechanism of colonisation, invasion etc., make it difficult to improve the quality of this protection at present. The protection observed in this study is discussed below.

D) Protection

Salmonellae produce two types of infection: a) general or systemic and b) enteric infection only (Parry and Porter 1981).

The oral vaccine for the protection against enteric infection had no effect on antibody production and protection of the offspring. As shown in this study, oral vaccination with killed vaccine alone failed to stimulate systemic antibody production and had no effect on protection of the chicks. The work of Truscott (1981) supports the first part of this view, as, when he vaccinated broiler chicks and directly challenged them with Salmonellae, he observed some degree of protection. Live vaccines may have a limited effect for the purpose of maternal antibody protection if introduced orally. Studies in Chapter 3 indicate that although there were some birds and eggs with high levels of antibody, equal numbers of birds and eggs were found to have no specific antibody. The protection afforded was not uniform among the chicks. The findings were, however, very interesting as gut colonisation was delayed for at least 8 hours when the challenge dose (0.8×10^3 CFU) was inoculated directly into the crop. SPF chicks inoculated with 1/4 of that dose (0.2×10^3 CFU) were colonised after 4 hours. When exposed to infection, some immune chicks were not colonised for at least a week after exposure. On this basis it is proposed that virulence and route of vaccination are important factors for the stimulation of antibody production and parenteral routes are preferable to oral. This supposition on the route of vaccination was confirmed by the studies in Chapter 4 for killed vaccine. As described there, subcutaneous injection of the killed vaccine produced antibody in all

vaccinated hens which was transferred to eggs and chicks. Knivett and Stevens (1971) showed that oral and subcutaneous routes are equally effective for live vaccines against direct challenge. The advantage of the subcutaneous route is that uniform antibody production results and it therefore appears preferable. The effect of the killed vaccine used in this study against direct challenge was not investigated. Suphabphant et al. (1983) used an oil-emulsion vaccine of killed RW16 strain of S. typhimurium subcutaneously and found that it can decrease Salmonella shedding following direct oral challenge with the same organism. The killed vaccine described in their study produced higher titres of antibody when compared to either oral or subcutaneous administration of the live G30D mutant of S. typhimurium. This not only supports the efficiency of the subcutaneous route but also shows that killed vaccine may be even more potent than live vaccine. Therefore to provide efficient protection the vaccine must be able to prevent or reduce gut colonisation as well as systemic infection. When the use of live vaccine is a risk for public health, killed vaccine is preferred and if the purpose of protection also includes the progeny, subcutaneous administration is superior to the oral route. After solving these problems the timing of vaccination is the next step. The choice is to vaccinate the chicks or the dam. Young chicks have been shown to be susceptible to infection with Salmonellae (Sadler 1969, Barrow et al. 1987a) and not to be capable of defending themselves as their immune system is not developed (Parry and Porter 1981). Therefore at this early stage of life, chicks are defenceless against exposure to infection and vaccination at this time cannot protect them very much. In this case maternal immunity seems to be the only remedy as it is for avian encephalomyelitis (Luginbuhl et al. 1984), avian infectious bronchitis (Hofstad 1984) and Newcastle disease (Lancaster 1964), etc.

This study demonstrated that maternally derived antibodies are capable of preventing systemic infection as well as delaying intestinal colonisation. These findings are in

agreement with the work of Thain et al. (1984) who found such protection in turkey poults and the work of Bigland et al. (1979) who used specific immunoglobulins for the control of Mycoplasma meleagridis in turkey embryos and poults.

Having in mind that vaccination of the dam is preferred to vaccination of the young chicks then the best time to vaccine the dam needs to be established. In this study vaccination prior to commencement of lay could produce levels of antibody which were transferred to the egg. Vaccination at this time is recommended for all chicken vaccines, because of the possible effect of vaccination stress on egg production during lay. Another reason for this timing was that in mammals plasma cells move from the site of original stimulation to the mammary gland before parturition. If this occurs in the hen by relocation of these cells to the oviduct before the start of lay, then the eggs will be equipped with specific antibodies which may help the embryos and young chicks. The presence of specific IgA and IgM in the oviduct of orally vaccinated hens may represent such an effect. Therefore vaccination of the dam for the purpose of producing passive immunity in chicks should begin a few weeks before lay. Earlier vaccination for the protection of the dams themselves may also necessary.

E) The role of immunoglobulins

The protective effect of maternally derived antibodies was shown in the challenge studies presented in Chapters 3 and 4. It was confirmed in Chapter 5, where the presence of specific IgA, IgM and IgG to S. typhimurium pt. 49 antigens in the sera of chicks was demonstrated by the ELISA test. The effect of each immunoglobulin was further studied by isolation and artificial transfer to SPF eggs. It was found that both IgG and IgA+IgM provided the best protection when injected into the yolk of embryos around day 10 of incubation. Bigland et al. (1979) showed that whole crude globulin fractions of either hyperimmune sera or sera obtained from the abattoir, and IgG or

IgM reduced the embryo mortality and the signs of the disease after hatch when injected into the yolk sac of eggs infected with M. meleagridis. IgG treatment was the most effective. The mechanism of action of these immunoglobulins was postulated at the end of Chapter 5. Additional evidence is provided by the work of Mukkur and Inman (1989) who demonstrated the role of specific IgG and IgM in the phagocytosis of S. typhimurium in vitro. IgM and IgG_2 showed opsonic activity which enhanced the phagocytosis of the organism by ovine mammary neutrophils. IgG_1 had no opsonic activity and IgM was superior to IgG_2. The opsonised organisms were killed in the neutrophils after ingestion.

CONCLUSIONS

In conclusion, the studies carried out in this thesis have shown that S. typhimurium pt. 49 can produce enteric as well as systemic infection in newly hatched SPF chicks by oral inoculation or exposure to infected chicks. The enteric infection results in colonisation of the gut with persistent shedding of the organism in the faeces at the level of 10^9 CFU/g up to at least 11 days after infection. Infected birds develop diarrhoea, depression, ruffled feathers, retardation of growth and death. Pericarditis, perihepatitis, inflammation of the gut mucosa and scattered necrotic foci in the liver provide histopathological evidence of systemic infection with the organism. The carrier birds were observed for at least 6 months after infection of the group. Gut colonisation occurred within 4 hours and systemic infection within 20 hours of infection.

Naturally infected chickens produced specific immunoglobulins to surface protein and LPS antigen of the organism in their sera, bile, oviductal washings, egg white and egg yolk. The levels of these immunoglobulins varied in different individuals. Some birds showed a marked difference from those in the SPF hens. Chicks derived from the immune eggs did not develop systemic infection. Gut colonisation was

delayed in these chicks.

Parenteral-oral vaccination of SPF hens at the point of lay resulted in specific immunoglobulin production in sera, eggs, bile and oviductal washing. Oral vaccination alone failed to produce any antibody in the sera of vaccinated hens. The protective immunity was transferred to eggs and the chicks derived from the eggs showed some degree of protection. Therefore vaccination could be an effective method in the control of poultry salmonellosis

Chicks derived from immune eggs acquired specific IgA, IgM and IgG in their sera with IgG reaching the highest level followed by IgA and IgM. This is the first report of presence of IgA and IgM in the serum of newly hatched chicks.

Immunoglobulin transfer to embryonated eggs resulted in the protection of newly hatched chicks with best protection provided by IgG followed by IgM+IgA when injected into the yolk sac.

The ELISA constructed was capable of identifying the presence of specific immunoglobulins in serum, bile, oviductal washings, egg yolk and egg white of the chicken with high sensitivity. This ELISA can be used routinely for monitoring infected flocks or monitoring the efficiency of the vaccination by using egg or serum.

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Appendix 1

Determination of the optimum LPS and surface protein concentrations was carried out by chequerboard titration of antigens and each reagent. Graphs of the relationship between the absorbance and the concentration of the reagent were prepared and the optimum concentration read from the graphs attached in this appendix. The titles of the graphs are as follows.

Figure I: The correlation between absorbance and surface protein antigen concentration using anti-chicken IgG on the positive standard serum.

II: The correlation between absorbance and LPS antigen concentration using anti-chicken IgG on the positive standard serum.

III: The correlation between absorbance and surface protein antigen concentration using anti-chicken IgA on the positive standard serum.

IV: The correlation between absorbance and LPS antigen concentration using anti-chicken IgM on the positive standard serum.

V: The correlation between absorbance and dilution of positive and negative standard sera using surface protein antigen and anti-chicken IgG antisera.

VI: The correlation between absorbance and dilution of positive and negative standard sera using LPS antigen and anti-chicken IgG antisera.

VII: The correlation between absorbance and dilution of positive and negative standard sera using surface protein antigen and anti-chicken IgA antisera.

VIII: The correlation between absorbance and dilution of positive and negative standard sera using LPS antigen and anti-chicken IgA antisera.

IX: The correlation between absorbance and dilution of positive and negative standard sera using surface protein antigen and anti-chicken IgM antisera.

X: The correlation between absorbance and dilution of positive and negative standard sera using LPS antigen and anti-chicken IgM antisera.

FIG. I:

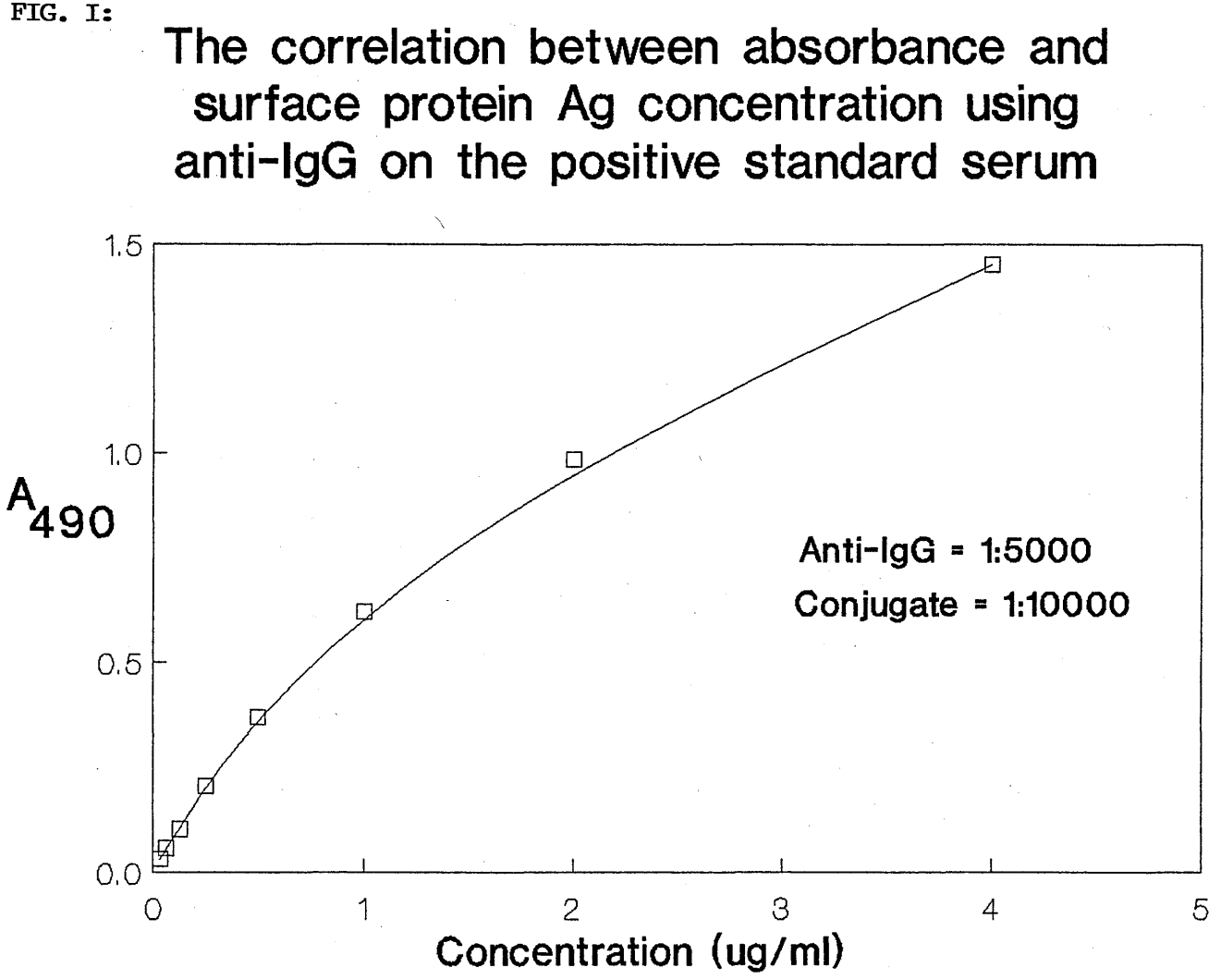

The correlation between absorbance and surface protein Ag concentration using anti-IgG on the positive standard serum
1.5
1.0
0.5
0.0
A490
Anti-IgG = 1:5000
Conjugate = 1:10000
0
1
2
3
4
5
Concentration (ug/ml)

FIG. II:

The correlation between absorbance and LPS Ag concentration using anti-IgG on the positive standard serum.

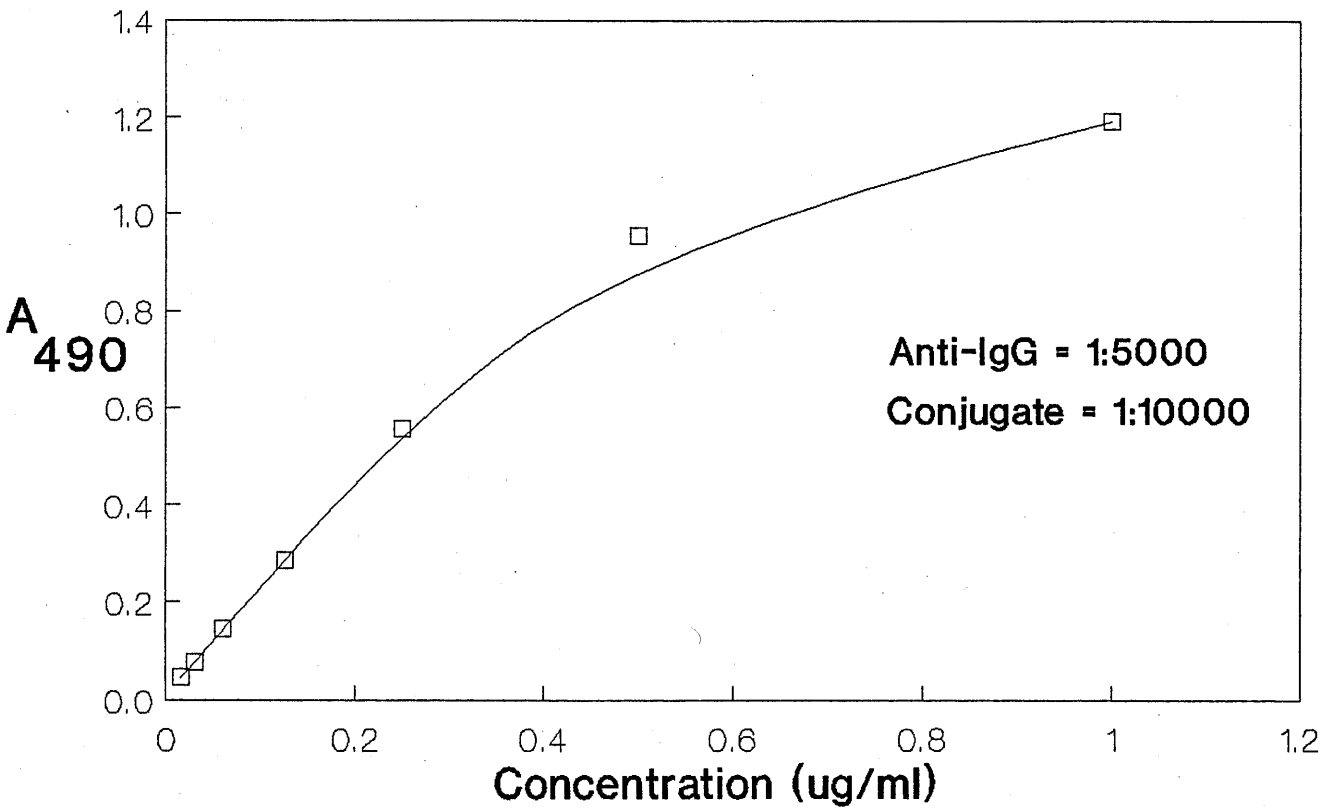


FIG. III:

The correlation between absorbance and surface protein Ag concentration using anti-IgA on the positive standard serum

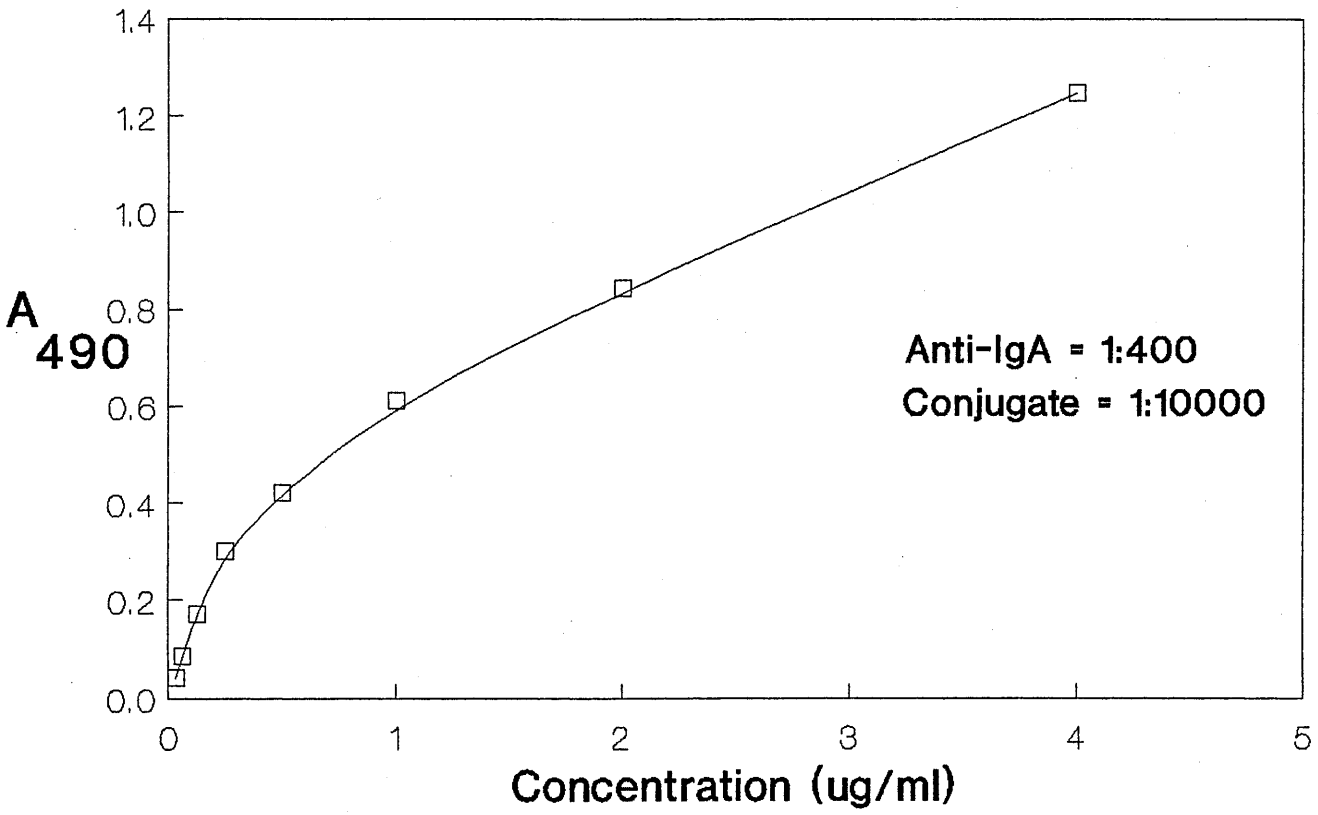


FIG. IV:

The correlation between absorbance and LPS Ag concentration using anti-IgM on the positive standard serum.

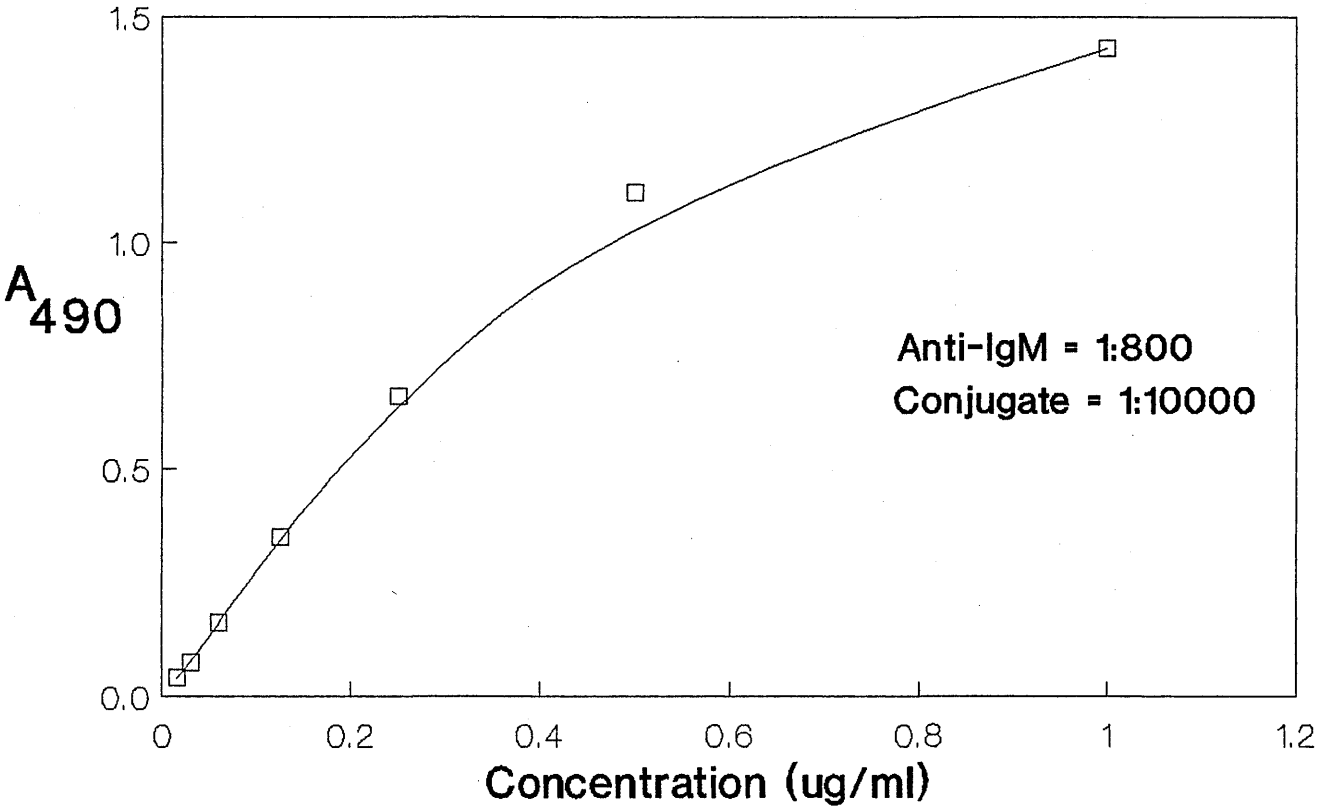


FIG. V:

The correlation between absorbance and dilution of positive and negative standard sera using surface protein antigen and anti-chicken IgG antisera.

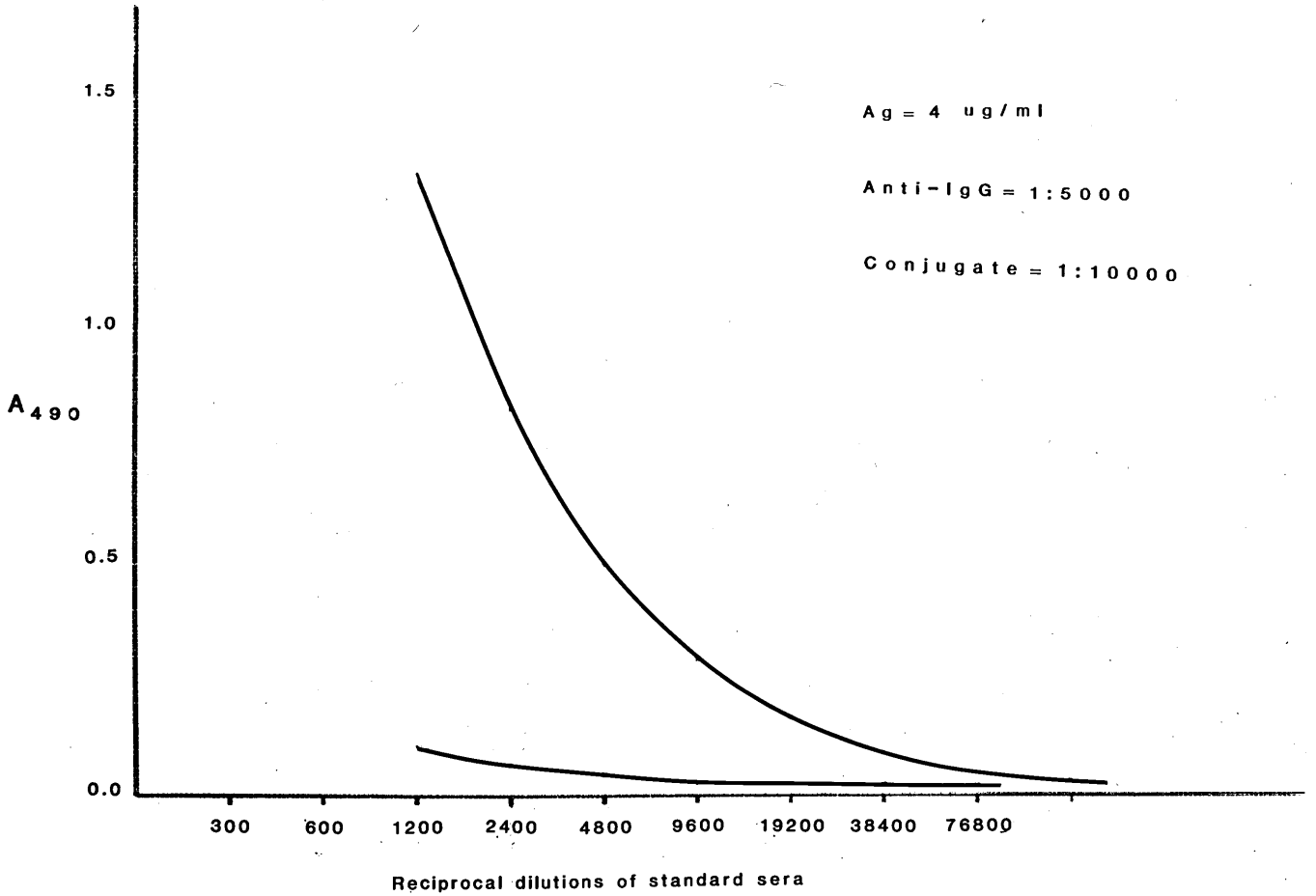


FIG. VI:

The correlation between absorbance and dilution of positive and negative standard sera using LPS antigen and anti-chicken IgG antisera.

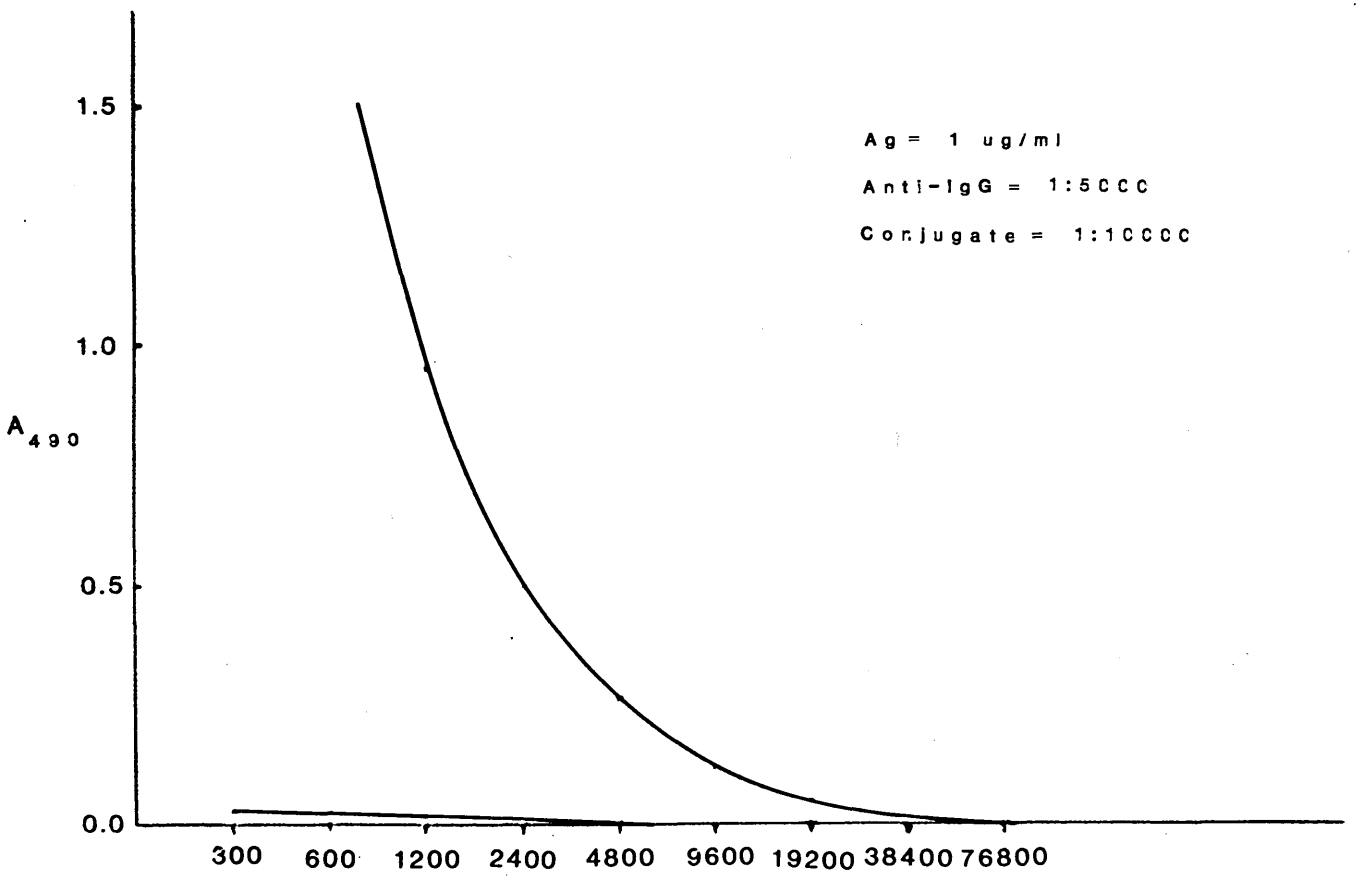


FIG. VII:

The correlation between absorbance and dilution of positive and negative standard sera using surface protein antigen and anti-chicken IgA antisera.

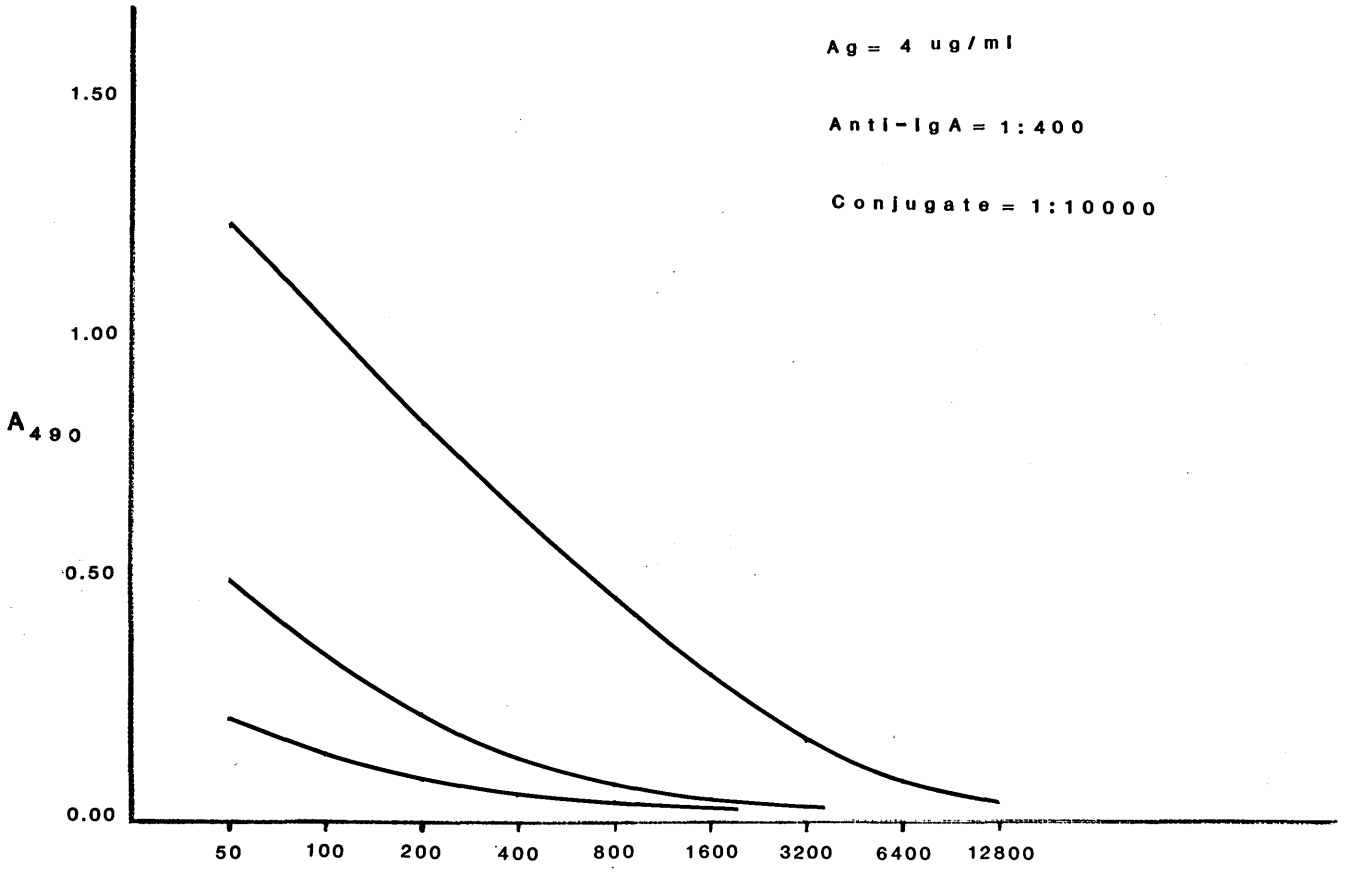


FIG. VIII:

The correlation between absorbance and dilution of positive and negative standard sera using LPS antigen and anti-chicken IgA antisera.

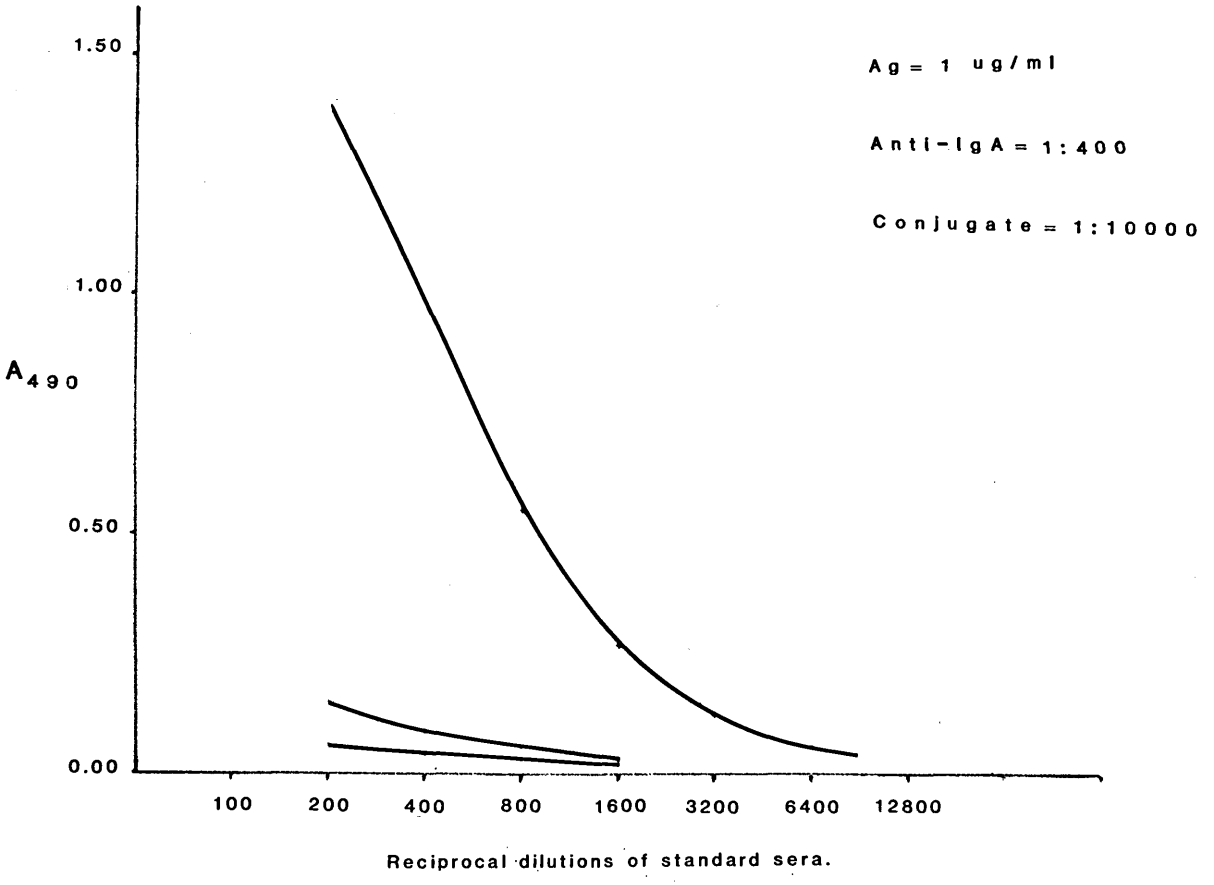


FIG. IX:

The correlation between absorbance and dilution of positive and negative standard sera using surface protein antigen and anti-chicken IgM antisera.

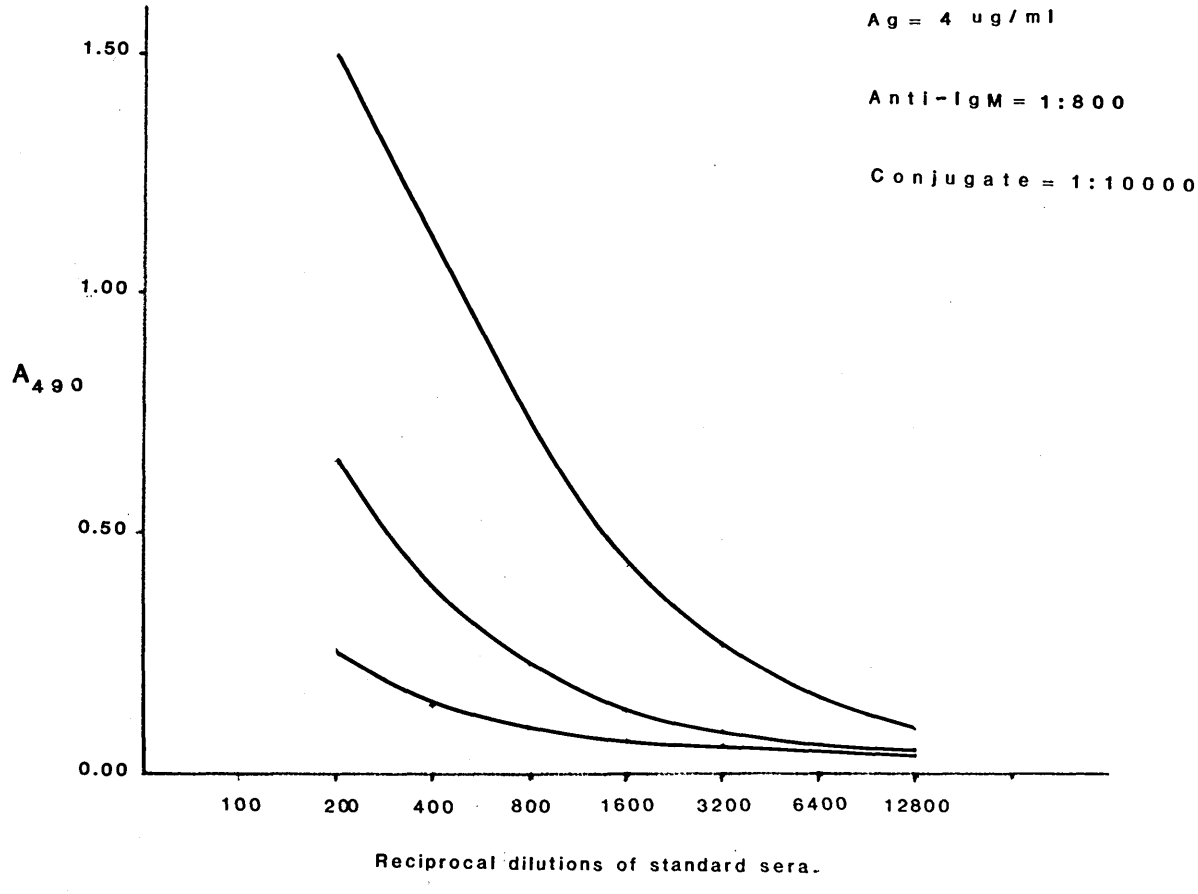
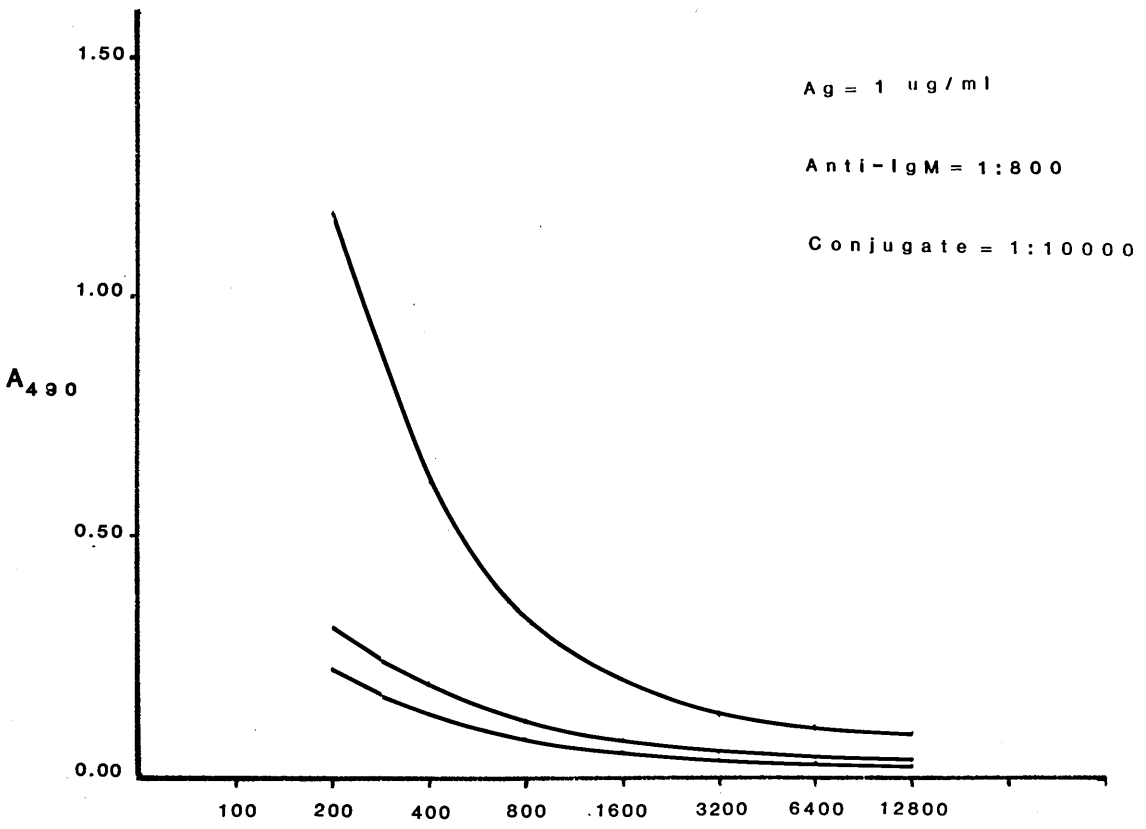


FIG. X:

The correlation between absorbance and dilution of positive and negative standard sera using LPS antigen and anti-chicken IgM antisera.



APPENDIX 2

The feeds used in this study were diet chicken starter, diet chicken grower and diet chicken breeder, supplied by Labsure, Lavender Mill, Manea, Cambridgeshire, England. The declared compositions of these feed are shown in this appendix.

Declared compositions of the rations used.

Composition	Starter	Grower	Breeder
Proximate analysis	%	%	%
Ash	5.7	5.0	11.1
Crude oil	2.5	2.3	2.3
Crude protein	18.4	14.9	14.8
Crude fibre	3.9	4.4	3.6
Calcium (as Ca)	0.99	0.81	3.1
Phosphorus (as P)	0.74	0.58	0.75
Sodium (Na)	0.20	0.19	0.20
Potassium (K)	0.83	0.69	0.66
Magnesium (Mg)	0.15	0.15	0.12
Chloride (Cl)	0.21	0.25	0.26
Carbohydrate	56	60	55
Starch	41	45	42
Digestible energy, MJ/Kg	13.2	12.9	12.3
Trace elements	**mg/Kg**	**mg/Kg**	**mg/Kg**
Manganese (Mn)	69.0	72.0	104.0
Copper (Cu)	13.0	12.0	12.0
Iron (Fe)	75.0	69.0	85.0
Iodine (added) (I)	0.50	0.50	1.0
Zinc (Zn)	56.0	59.0	93.0
Selenium (Se)	0.21	0.18	0.18
Cobalt (Co)	---	---	0.5
Amino acids	**g/Kg**	**g/Kg**	**g/Kg**
Threonine	6.8	5.3	5.3
Glycine	8.0	6.5	6.3
Valine	9.3	7.5	7.4
Cystine	3.1	2.7	2.6

Declared compositions ..... continued:

Methioine	3.9	3.1	3.4
Isoleucine	8.4	6.4	6.5
Leucine	14.6	11.4	10.9
Tyrosine	7.1	5.6	5.4
Phenylalanine	9.3	7.4	7.4
Lysine	9.4	6.8	6.9
Histidine	4.6	3.7	3.6
Arginine	12.1	9.4	9.5
Tryptophane	2.3	1.8	1.8
Vitamins	**per Kg**	**per Kg**	**per Kg**
Vitamin A (Retinol) iu	10000	10000	16000
Vitamin D3 (Cholecalciferol) iu	3000	3000	5000
Vitamin E (Alpha Tocopherol) iu	12	14	31
Vitamin K3 added mg	2	2	3
Vitamin B1 (Thiamine) mg	4	5	5
Vitamin B2 (Riboflavin) mg	6	6	11
Vitamin B6 (Pyridoxine) mg	6	5	9
Niacin (Nicotinic acid) mg	29	27	57
Pantothenic acid mg	19	20	25
Folic acid mg	0.7	0.7	3
Vitamin B12 (Cyanocobalamin) mg	0.006	0.006	0.015
Choline mg	1300	1200	1270
Pellet size mm	3.2	3.2	3.2

All diets were declared to be free from coccidiostat and growth promoters and irradiated at 5 M Rad.

Appendix 3

All of the SPF eggs and chickens used were purchased from Wickham Laboratories (SPF Farms) Ltd., Winchester Road, Wickham, Hants, England. Every consignment of eggs or birds delivered was accompanied by a certificate indicating the health status of the flock of origin of the consignment and including the most recent tests carried out on that flock. A copy of one of these certificates is included in this appendix.

WICKHAM
laboratories (S.P.F. Farms) limited
Winchester Road
WICKHAM
HANTS PO17 5EU
ENGLAND

telephone (0329) 832511
telex 86184 WIKLAB G

Farm address
Torbay Farm, Lower Upham, Southampton
telephone Durley (04896) 515

Correspondence and Enquiry to Wickham
except where otherwise stated

IT IS HEREBY CERTIFIED that the Testing Certificate on the reverse hereof relates to the following consignment:-

Flock: TORBAY 7

Date of Consignment: 31.5.88

Number of eggs: 60

Customer's Name and Address

DEPARTMENT OF VETERINARY PATHOLOGY
UNIVERSITY OF GLASGOW VETERINARY SCHOOL
BEARSDEN ROAD
BEARSDEN
GLASGOW G61 1QH

.....
Wickham Laboratories (S.P.F. Farms) Ltd.

WICKHAM LABORATORIES LIMITED
(Testing laboratory)

SPECIFIC PATHOGEN FREE FLOCK MONITORING DATES
FLOCK REFERENCE: TORBAY 7

IT IS HEREBY CERTIFIED THAT THIS FLOCK HAS BEEN TESTED IN ACCORDANCE WITH THE FOLLOWING SCHEDULE AND HAS GIVEN NEGATIVE RESULTS IN EACH CASE UNLESS OTHERWISE STATED
THIS FLOCK IS KNOWN TO BE POSITIVE TO DISEASES MARKED *

INFECTIOUS AGENT	ANTIGEN	TEST TYPE	SAMPLING DATES 1	2	3	4	5	6	7	8
Mycoplasma Synoviae	WVU-1853	S.P.A.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Mycoplasma Gallisepticum	S6	S.P.A.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Salmonella Pullorum	STD/VAR	S.P.A.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Influenza Type A	WILSON	A.G.P.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Marek's Disease	A	A.G.P.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Infectious Bursal Disease	52/70	A.G.P.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Adenovirus	PHELPS	A.G.P.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Reovirus	RT6	A.G.P.T.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Newcastle Disease	F	H.A.I.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Infectious Bronchitis	M 41	H.A.I.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Adenovirus	127	H.A.I.	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	18/04/88
Avian Lymphoid Leucosis A	RSV(RAV 1)	S.N.	10/08/87	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88
Avian Lymphoid Leucosis B	RSV(RAV 2)	S.N.	10/08/87	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88
Avian Infectious Laryngotracheitis	96	S.N.	08/09/86	08/12/86	09/03/87	08/06/87	14/09/87	07/12/87	07/03/88	
Avian Reticuloendotheliosis	HPRS-1	S.N.	19/05/86	11/08/86	10/11/86	09/02/87	11/05/87	10/08/87	09/11/87	09/02/88
Avian Adenovirus		S.N.	19/05/86	01/07/86	13/10/86	12/01/87	13/04/87	13/07/87	12/10/87	11/01/88
Avian Encephalomyelitis	VAN ROEKEL	F.E.S.	09/09/87	28/10/87	11/11/87	09/12/87	27/01/88	10/02/88	09/03/88	13/04/88
Other Salmonella Species	N.A.	Selective Enrichment	14/09/87	12/10/87	09/11/87	07/12/87	11/01/88	08/02/88	07/03/88	19/04/88
Fowl Pox			21/09/87	21/10/87	20/11/87	30/12/87	22/01/88	26/02/88	28/03/88	19/04/88

The flock is sampled at > 5% in any given month except:
Fowl Pox = Clinical examination
Avian Infectious Laryngotracheitis = > 5% in any three months
Avian Reticuloendotheliosis = > 5% in any three months
Avian Adenovirus (S.N.) = > 5% in any three months
Avian Encephalomyelitis = > 50 embryos in any month
Other Salmonella Species = 1 composite faecal sample per 2 cages (maximum 10 samples) in any month

A.G.P.T. = Agar Gel Precipitation Test
H.A.I. = Haemagglutination Inhibition
S.N. = Serum Neutralisation
S.P.A.T. = Serum Plate Agglutination Test
F.E.S. = Flock Embryo Susceptibility

Issue number:23 /APR

Date of issue:29th April, 1988

Signed Virology:

K. W. Mayerle

Signed Bacteriology:

W A Barker

WICKHAM LABORATORIES LTD

APPENDIX 4

The mean levels of specific IgA, IgM and IgG in the sera of the parenterally-orally and orally vaccinated chickens are shown in Figs. 35-40 in the form of curves. The individual levels are given in Tables I-VI in this appendix under the same titles. The figure shown in week 49 for hen 3 is the result of the ELISA test on the serum at killing time (week 47). The titles of these tables are as follows:

Table I: Levels of serum IgG against surface protein antigen in vaccinated chickens.

II: Levels of serum IgG against LPS antigen in vaccinated chickens.

III: Levels of serum IgM against surface protein antigen in vaccinated chickens.

IV: Levels of serum IgM against LPS antigen in vaccinated chickens.

V: Levels of serum IgA against surface protein antigen in vaccinated chickens.

VI: Levels of serum IgA against LPS antigen in vaccinated chickens.

Table I (Data for Fig. 35)

Levels of serum IgG against surface protein antigen in vaccinated chickens.

Age in weeks	The chicken No. 1	2	3	4	5	6	7
17*	1200**	600	1200	1200	1200	1200	1200
21+	600	38400	38400	9600	9600	38400	1200
23	1200	19200	76800	19200	38400	38400	1200
25	1200	38400	76800	38400	38400	76800	1200
27	1200	38400	38400	38400	19200	76800	1200
29	1200	19200	19200	38400	9600	76800	1200
33	600	19200	38400	19200	9600	76800	1200
36	600	19200	38400	19200	9600	38400	1200
40	600	38400	38400	38400	38400	76800	1200
44	600	38400	76800	38400	38400	76800	1200
49	600	38400	76800	38400	38400	76800	1200
52	--	--	--	--	--	--	2400
59	600	38400	--	38400	38400	38400	--

* = Pre inoculation bleed.

** = Reciprocal dilution of serum.

+ = First bleed after vaccination at week 19.

Table II (Data for Fig. 36)

Levels of serum IgG against LPS antigen in vaccinated chickens.

Age in weeks	The chicken No. 1	2	3	4	5	6	7
17*	1200**	600	1200	1200	1200	600	300
21+	600	9600	4800	9600	9600	4800	600
23	1200	9600	9600	9600	9600	4800	600
25	1200	19200	19200	19200	9600	19200	600
27	1200	19200	9600	19200	4800	38400	600
29	1200	19200	9600	19200	4800	19200	600
33	1200	19200	9600	19200	4800	19200	600
36	1200	19200	9600	9600	4800	19200	600
40	1200	38400	19200	38400	19200	38400	1200
44	1200	19200	9600	19200	9600	19200	1200
49	1200	19200	9600	19200	9600	19200	1200
52	--	--	--	--	--	--	1200
59	1200	19200	--	19200	9600	19200	--

* = Pre inoculation bleed.

** = Reciprocal dilution of serum.

+ = First bleed after vaccination at week 19.

Table III (Data for Fig. 37)

Levels of serum IgM against surface protein antigen in vaccinated chickens.

Age in weeks	The chicken No. 1	2	3	4	5	6	7
17*	1600**	800	800	800	800	1600	800
21+	1600	25600	12800	12800	12800	12800	1600
23	1600	6400	6400	3200	6400	6400	3200
25	1600	12800	12800	12800	6400	25600	3200
27	3200	12800	12800	12800	6400	25600	3200
29	3200	6400	6400	12800	6400	12800	3200
33	3200	6400	6400	6400	6400	12800	3200
36	1600	6400	6400	6400	6400	12800	3200
40	1600	12800	12800	12800	12800	25600	3200
44	1600	12800	6400	12800	6400	25600	3200
49	1600	12800	6400	12800	6400	25600	3200
52	--	--	--	--	--	--	3200
59	1600	12800	--	12800	6400	12800	--

* = Pre inoculation bleed.

** = Reciprocal dilution of serum.

+ = First bleed after vaccination at week 19.

Table IV (Data for Fig. 38)

Levels of serum IgM against LPS antigen in vaccinated chickens.

Age in weeks	The chicken No.						
	1	2	3	4	5	6	7
17*	1600**	800	800	800	1600	1600	800
21+	1600	25600	6400	6400	12800	12800	1600
23	1600	3200	3200	3200	3200	6400	3200
25	1600	12800	6400	12800	6400	25600	3200
27	1600	12800	6400	12800	6400	25600	3200
29	1600	6400	3200	6400	6400	12800	3200
33	1600	6400	3200	6400	6400	12800	3200
36	1600	6400	3200	6400	6400	12800	3200
40	1600	12800	6400	12800	12800	25600	3200
44	1600	6400	3200	12800	6400	25600	3200
49	1600	6400	3200	6400	6400	25600	3200
52	--	--	--	--	--	--	3200
59	1600	6400	--	6400	6400	25600	--

* = Pre inoculation bleed.

** = Reciprocal dilution of serum.

+ = First bleed after vaccination at week 19.

Table V (Data for Fig. 39)

Levels of serum IgA against surface protein antigen in vaccinated chickens.

Age in weeks	The chicken No. 1	2	3	4	5	6	7
17*	400**	400	400	400	400	400	400
21+	800	12800	12800	6400	6400	6400	800
23	800	6400	12800	3200	6400	6400	1600
25	800	6400	12800	6400	6400	12800	1600
27	1600	6400	12800	6400	6400	12800	1600
29	1600	6400	6400	6400	3200	12800	1600
33	800	3200	6400	6400	3200	6400	1600
36	800	3200	6400	6400	3200	6400	1600
40	800	12800	12800	12800	12800	12800	1600
44	800	6400	6400	6400	12800	12800	1600
49	800	6400	6400	6400	12800	12800	1600
52	--	--	--	--	--	--	1600
59	800	6400	--	6400	6400	12800	--

* = Pre inoculation bleed.

** = Reciprocal dilution of serum.

+ = First bleed after vaccination at week 19.

Table VI (Data for Fig. 40)

Levels of serum IgA against LPS antigen in vaccinated chickens.

Age in weeks	The chicken No. 1	2	3	4	5	6	7
17*	1600**	800	800	800	800	1600	800
21+	1600	6400	3200	3200	6400	6400	1600
23	1600	1600	3200	1600	3200	3200	1600
25	1600	3200	6400	6400	6400	12800	1600
27	1600	3200	6400	6400	3200	12800	1600
29	1600	3200	3200	6400	3200	12800	1600
33	1600	3200	3200	6400	3200	12800	1600
36	1600	3200	3200	6400	3200	12800	1600
40	1600	6400	6400	12800	12800	12800	1600
44	1600	3200	6400	6400	6400	12800	1600
49	1600	3200	6400	6400	6400	12800	1600
52	--	--	--	--	--	--	1600
59	1600	3200	--	6400	6400	12800	--

* = Pre inoculation bleed.

** = Reciprocal dilution of serum.

+ = First bleed after vaccination at week 19.