EVALUATION OF PATHOGENICITY AND ANTIGENIC RELATIONSHIP OF *Salmonella* ISOLATES FROM POULTRY

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled **"EVALUATION OF PATHOGENICITY AND ANTIGENIC RELATIONSHIP OF *Salmonella* ISOLATES FROM POULTRY"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled ""**EVALUATION OF PATHOGENICITY AND ANTIGENIC RELATIONSHIP OF *Salmonella* ISOLATES FROM POULTRY**" is a record of research work done independently by **Sunil G.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Sunil G

TO

MY BELOVED

AMMA

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Introduction

1. INTRODUCTION

India, with developing economy, depends mostly on agriculture and animal husbandry for generating national income. The ever-increasing demand of egg and chicken meat, both from inside and outside the country, has triggered the rapid development of poultry farming in India. This production oriented intensive management has led to increased incidence of various diseases. Among them fowl typhoid (FT), caused by *Salmonella gallinarum* (*S. gallinarum*) is wide spread and is one of the most important egg-borne bacterial diseases.

Salmonellosis in poultry is a disease of major economic importance, distributed world wide and is responsible for substantial losses to the poultry industry, by causing heavy mortality, morbidity and loss of production. (Rathore *et al.*, 2005). The outbreak of FT is characterized by increased mortality, anorexia, greenish-yellow diarrhea and drop in egg production. Sub acute outbreak can occur and egg transmission may lead to increase in dead or weak chickens (Lee *et al.*, 2003). Although control programmes including vaccination have largely controlled the disease in Europe and North America, it remains an enigma to developing poultry industries in Asia and South America. (Wigley *et al.*, 2005)

Domestic poultry constitute the single largest reservoir of *Salmonella* in nature. More than 50 per cent of isolates of *Salmonella* from sources other than human being are from poultry and poultry products. *Salmonella* is worldwide in distribution and has been found extensively in all poultry producing areas of the world and India is no exception. Kerala is also not free of *Salmonella* infection. Incidences of salmonellosis are frequently reported in chicken and other birds of this state (Mary, 2000). When different samples from 155 birds were examined for *Salmonella* by genus specific and serovar specific polymerase chain reaction (PCR), 46 were found to be positive (Muthuramalingam, 2005).

Avian salmonellosis is thus a menace to poultry industry, causing huge economic losses. The control of this disease is extremely difficult since salmonellae are environmental pathogens and could survive in poultry shed dust, soil, floor, walls and air for pretty long time. Treatment is also difficult because of the chronic nature of the disease; also *Salmonella* isolates are showing continuously evolving patterns of resistance to antimicrobials (Lee *et al.*, 2003).

Hence, the only feasible method for the control of this disease is by vaccination. But the presence of large number of serovars is an important stumbling block in this regard, since the antigenic homogeneity/heterogeneity of the vaccine strain with the field strain greatly determines the efficacy of the developed vaccine. This study will therefore help to understand the role of salmonellae isolated from birds of different parts of Kerala in the causation of disease, which in turn will help to adopt proper control measures.

The present study is intended to throw light on the following aspects of FT

1) The virulence of *Salmonella* isolates obtained from poultry of different regions of Kerala.

2) The pathogenicity and antigenic relationship of the local isolates with that of a *S. gallinarum* reference strain/known strain and

3) The antigenic homogeneity/heterogeneity of these isolates.

Review of Literature

2. REVIEW OF LITERATURE

2.1 FOWL TYPHOID

Fowl typhoid (FT) continues to be a menace for the poultry industry all over the world, including Indian sub-continent. The disease is caused by infection with *Salmonella enterica* serovar Gallinarum in chickens and is usually observed in adult birds, although the agent is very pathogenic for chickens of all ages (Pomeroy and Nagaraja, 1991).

Most of the salmonellae are marginally pathogenic in birds. *Salmonella gallinarum* and *Salmonella pullorum* (*S. pullorum*) could be highly virulent in poultry flocks, despite the fact that they are neither motile nor hemagglutinating. Both were avirulent in mammalian animal models (Snoeyinbos, 1991).

Salmonella belongs to the family *Enterobacteriaceae*, non-sporing Gram-negative rods that are non-acid fast and facultatively anaerobic. Most of the serotypes are motile with peritrichous flagella, but *S. gallinarum* and *S. pullorum* are non-motile. Fowl typhoid had been reported in turkey flocks, ducks, pheasants, guinea fowl, pea fowl, goose and quails, apart from chicken. The organism usually infected growers or adult birds, although chicks could be affected (Wray *et al.*, 1996).

Lee *et al.* (2003) reported FT as a septicemic disease of domestic birds caused by *S. gallinarum*. The outbreak of this disease was characterized by increased mortality, anorexia, greenish-yellow diarrhea, and a drop in egg production. Sub acute outbreak could also occur, and egg transmission might lead to increase in dead or weak chickens.

Salmonellosis, one of the major diseases of economic importance, is distributed world wide. It is responsible for substantial losses to the poultry industry in terms of heavy mortality, morbidity and loss of production (Rathore *et al.*, 2005).

Wigley *et al.* (2005) reported that although control programmes including vaccination had largely prevented the disease in Europe and North America, it remained of high economic importance to developing poultry industries in Asia and South America.

2.2 INCIDENCE

2.2.1 Incidence of Avian Salmonellosis in India

Saxena *et al.* (1983) reported isolation of 445 *Salmonella* strains distributed into 28 different serotypes, from poultry received for identification and serotyping at the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, during 1974-82. The most frequently isolated serotype was were *S. gallinarum* (218 strains). The largest numbers of strains (245) were obtained from Pune in Maharashtra.

Gupta and Verma (1989) identified 170 *Salmonella* strains belonging to 13 serotypes and eight rough variants from various avian sources, during 1977 to 1986, at National Salmonella Centre, Indian Veterinary Research Institute (IVRI), Izatnagar. Of these, *S. gallinarum* was the predominant serotype constituting 25.29 per cent. Of the total 43 referred strains of *S. gallinarum*, 95.5 per cent of the strains were isolated from chickens and 4.5 per cent from pigeon, indicating the possible role of pigeon in the transmission of the disease. This study also indicated the prevalence of *S. gallinarum* infection in West Bengal, Uttar Pradesh, Assam, Maharashtra, Gujarat and Karnataka.

A study was conducted by Verma and Gupta (1997) to find out prevalence of *Salmonella* serotypes of avian origin. During the period from 1987 to 1995, 338 *Salmonella* strains belonging to 21 serovars and one monophasic variant were identified at the National Salmonella Centre (veterinary), Uttar Pradesh. *Salmonella gallinarum* was the predominant serotype constituting 32.25 per cent of the referred strains. Most of the isolations were from chicken (99.8 per cent) and one strain was recovered from pigeon. The organism showed wide prevalence in India with maximum isolates from Bangalore (30/338).

Verma *et al.* (2000) identified various *Salmonella* cultures obtained from different places in India during 1994-96 at IVRI, Izatnagar. A total of 193 *Salmonella* cultures belonging to 29 serovars were typed. Majority of the isolates (94) were of avian origin. *Salmonella enteritidis* (*S. enteritidis)* was found to be the most predominant serovar, followed by *Salmonella indiana* (*S. indiana)*, *Salmonella typhimurium* (*S. typhimurium)*, *S. gallinarum*, *Salmonella paratyphi* B (*S. paratyphi* B), *Salmonella eastbourne* (*S. eastbourne)* and *Salmonella dublin* (*S. dublin)*. Rare *Salmonella* serovars (*viz.*, Albany, Agona, Catanzaro and Teko) were also recorded from various sources in India.

Shukla and Singh (2001) isolated 19 strains of *Salmonella* from 220 poultry samples consisting of 37 samples of fresh fecal droppings, 23 old fecal materials, 67 samples from outer surfaces of eggs, 67 from inner shell membrane of eggs and 26 samples of broken eggs collected from Veterinary College Poultry Farm, Mathura. The relative proportion of *Salmonella* isolations were fresh fecal droppings (5.4 per cent), old fecal droppings (4.34 per cent), eggshell outer surface (5.97 per cent), shell membrane surface (11.94 per cent) and broken eggs (15.38 per cent).

Rahman *et al.* (2002) reported an outbreak of salmonellosis in a layer farm at Assam. The organism associated was identified as *S. typhimurium.*

Muniyellappa *et al.* (2003) isolated *S. gallinarum* from 48 out of 69 liver samples of birds died of FT at different broiler farms in and around Bangalore.

A total of 23 *Salmonella* isolates were recovered from 40 different samples collected during different disease outbreaks. Of these, fifteen, five and three isolates were recovered from Karnataka, Maharastra and Tamil Nadu respectively. The serotypes isolated were *S. gallinarum*, *S. enteritidis*, *S. typhimurium* and *Salmonella worthington (S. worthington)*. The most predominant serotype was *S. gallinarum* (69.6 per cent), followed by *S. enteritidis* (21.7 per cent) (Prakash *et al.*, 2005).

Isolation of *Salmonella* serotypes was attempted from 125 fresh and 50 frozen chicken samples collected from poultry shops, commercial farms and retail outlets in Srinagar. *Salmonella* was recovered from 2.4 per cent and four per cent samples of fresh and frozen chicken, respectively. Five isolates of salmonellae were typed into two serotypes of which *S. enteritidis* and *S. reading* constituted 80 and 20 per cent, respectively (Willayat *et al.*, 2006).

2.2.2 Incidence of Avian Salmonellosis in Kerala

Krupeshsharma and Rajan (1996) reported that salmonellosis was prevalent in quails of Kerala and the organism isolated was *S. typhimurium.*

Mini *et al.* (2005) reported isolation and identification of *S. enteritidis* with an antigenic structure 9, 12: g, m from two, two week-old dead broiler chicks causing heavy mortality of birds in the flock.

Muthuramalingam (2005) examined 155 clinical samples from suspected cases of salmonellosis from ailing birds from different parts of Kerala. Forty six were positive for salmonellae and among them, 15 were characterized as

S. gallinarum. He also reported that the most prevalent *Salmonella* serotype in Kerala was *S. gallinarum*.

Salmonella gallinarum could be regularly isolated from birds brought to Department of Veterinary Microbiology, COVAS, Mannuthy, Kerala for disease investigation (Nair, G.K. 2007. personal communication; unreferenced).

2.3 ANTIBIOTIC SENSITIVITY TEST (ABST) OF SALMONELLAE

Poppe *et al.* (1995) conducted a study to determine the antibiotic resistance and biochemical characteristics of 2690 *Salmonella* strains belonging to 52 serovars isolated from environmental and feed samples from 270 turkey flocks in Canada. They found that the different *Salmonella* serovars varied widely in number of resistant strains of a particular serovar and in type of drug resistance. Resistance of *Salmonella* strains to aminoglycoside antibiotics varied widely; none of the strains were resistant to amikacin, 14.2 per cent were resistant to neomycin, 25.8 per cent were resistant to gentamicin, and 27.7 per cent of the strains were resistant to kanamycin. Most strains (97.6 per cent) were resistant to the aminocyclitol and spectinomycin. Regarding resistance to the β–lactam antibiotics, 14.3 per cent and 14.4 per cent of the strains were resistant to ampicillin and carbenicillin, respectively, whereas only 0.2 per cent of the strains were resistant to cephalothin. None of the strains were resistant to the fluoroquinolone, ciprofloxacin or to polymyxin B. Resistance to chloramphenicol and nitrofurantoin was found in 2.4 and seven per cent of the strains respectively. Only 1.7 per cent of the strains were resistant to the trimethoprim-sulfamethoxazole combination, whereas 58.1 per cent were resistant to sulfisoxazole. Thirty-eight per cent of the strains were resistant to tetracycline.

Rahman *et al.* (1997) isolated six *S. gallinarum* and two *S. indiana* isolates from a disease outbreak in a broiler farm in Assam. The ABST of these salmonellae revealed similar type of antibiotic susceptibility profile. All the eight

isolates were sensitive to chloramphenicol, gentamicin and norfloxacin, followed by seven to nalidixic acid, six strains each to trimethoprim, streptomycin and kanamycin and three strains to tetracycline.

Joshi *et al.* (2000) reported an outbreak of FT in a commercial layer farm in Madhya Pradesh. *Salmonella gallinarum* was isolated from clinical samples and the isolates showed maximum sensitivity to chloramphenicol, followed by gentamicin, polymyxin-B, neomycin, ciprofloxacin and streptomycin. They were resistant to tetracycline, chlortetracycline, amoxycillin, ampicillin and doxycycline. The sensitivity pattern indicated the development of resistance for the antibiotics which were in regular use for the treatment and prophylaxis of bacterial infections in poultry.

Shukla and Singh (2001) isolated 19 strains of *Salmonella* from 220 poultry samples. These isolates were subjected to ABST and they found that most of the isolates were highly sensitive to streptomycin, tetracycline, ciprofloxacin and kanamicin. Strains tested were completely resistant to penicillin-G and amoxycillin. Rest of the antibacterial agents like chloramphenicol, erythromycin, nystatin, nitrofurantoin, bacitracin, sulphaphenazole and furazolidone were either ineffective or just sensitive.

Salmonella typhimurium isolates showed high susceptibility to amikacin, chloramphenicol, gentamicin, norfloxacin, cefotaxim, mezlocillin and sulphamerazine, but were resistant to ampicillin, oxytetracycline and streptomycin (Rahman *et al.*, 2002).

Lee *et al.* (2003) studied antibiotic sensitivity pattern of 258 *S. gallinarum* isolates during 1995 to 2001 from Korea. The antibiogram of these isolates using disks containing ampicillin, amoxycillin/clavulanic acid, gentamicin, kanamycin, enrofloxacin, ciprofloxacin, norfloxacin, ofloxacin, tertacycline, oxyteracycline, colistin and sulfamethoxazol/trimethoprim revealed that all of them were

susceptible to all the antimicrobial agents tested except for tetracycline and oxytetracycline. They also noticed that vast majority of isolates obtained during 2001 showed a reduced susceptibility to antimicrobials tested.

Sujatha *et al.* (2003) reported isolation of six *S. gallinarum* strains from 21 positive reactor birds of different poultry farms located in and around Hyderabad and Secunderabad. Antibiogram of the isolates revealed 100 per cent sensitivity to ciprofloxacin, amoxycillin, chloramphenicol and ampicillin while furazolidone, erythromycin and cloxacillin were least effective. They suggested that the efficacy of these drugs might be related to the less frequent use of them for treatment and control of bacterial diseases.

Saxena *et al.* (2005) carried out antibiogram for 24 strains of *Salmonella* and found that strains lacking the plasmid also showed resistance for many antibiotics including ciprofloxacin, lincomycin, tetracycline, nitrofurazolidone and erythromycin.

Willayat *et al.* (2006) conducted *in vitro* antibiotic sensitivity of salmonellae isolated from fresh and frozen chicken in Srinagar. They found 100 per cent sensitivity to nalidixic acid, chloramphenicol, amoxycillin, norfloxacin, ciprofloxacin and resistance to erythromycin. Seventy per cent of *S. enteritidis* isolates were sensitive to ampicillin, cephalosporin and pefloxacin but *S. reading* isolates were resistant to cephalosporin, co-trimoxazole and tetracycline.

2.4 VIRULENCE FACTORS OF SALMONELLAE

Poppe and Gyles (1987) examined a collection of 185 isolates of 34 serovars of *Salmonella* from avian sources for plasmids, drug resistance, biochemical properties, serum resistance and virulence. They found that there were no indications that strains recovered from outbreaks were different from that normally seen in the intestine. Therefore, they concluded that the emergence of

virulent strains was not the result of acquisition of special virulence characteristics by the organisms, but rather was the result of some deficiency on the part of the host.

Barrow and Lovell (1989) reported considerable degree of functional homology between plasmids of *S. gallinarum*, *S. pullorum*, and *S. typhimurium*. Plasmid curing was associated with loss or reduction of virulence, which could be completely restored by re-introduction of the virulence associated plasmids from heterologous *Salmonella* serotypes or biotypes in addition to plasmid from homologous strain. But these virulence associated plasmids could not restore virulence in an avirulent *Escherichia coli* (*E. coli*) K-12, indicating the importance of chromosomal determinants in the full expression of virulence.

Barrow (1990) observed that virulence plasmid cured strains of *S. gallinarum* even though less virulent were also less immunogenic than virulence plasmid possessing 9R vaccine strain. He suggested that virulence plasmid contributed to complete immunogenicity.

Anjanappa *et al.* (1994) conducted studies on the isolation of plasmids from 25 field strains of *S. gallinarum* and their derivatives by alkaline lysis method. Majority of the strains exhibited a single plasmid of molecular weight of 56 Md, except three strains which had a plasmid of 2 Md, in addition to the 56 Md plasmid. They also found that one strain having this 56 Md plasmid was sensitive to all the antimicrobial agents tested. Three strains without plasmids showed multiple drug resistance and they opined that this could be due to the transposone(s) or episome(s) or mutations on chromosomal genes due to antibiotic pressure.

Barrow *et al.* (1994) demonstrated the host specificities of host-specific *Salmonella* serotypes by experimental infection of chickens, mice and other laboratory animals. Following oral inoculation, four strains of *S. gallinarum* and

two *S. pullorum* strains, isolated from diseased poultry, were more virulent for chicken than for mice, and in contrast, four strains each of *Salmonella choleraesuis* (*S. choleraesuis)* and *S. dublin* isolated from diseased pig and cattle respectively were more virulent to mice than chicken. The virulence of host-specific serotypes following oral inoculation was similar to that seen following intravenous or intramuscular inoculation supporting the contention that host specificity in the strains studied is primarily expressed in the differential ability to multiply in tissues, particularly those of reticuloendothelial system, in immunologically mature animal. This was evident by the fact that there was greater difference in the ability to survive and multiply in the visceral organs compared to distal alimentary tract, once invasion had occurred, which correlated with the virulence for the host species involved. The serum resistance of the strains tested implied not only that serum sensitivity did not play a part in determining host specificity but also that serum sensitivity would be no barrier to extra cellular bacterial multiplication if it was involved.

Ghosh *et al.* (1995) reported loss or decrease in virulence of *Salmonella abortus equi* (*S. abortus equi)* by cultivating the organisms in the medium containing ethidium bromide or growing at elevated temperature (42-43°C). The variants either were completely devoid of or decreased in lethality, when tested in Swiss albino mice by inoculating 18 h broth culture by subcutaneous route or by intraperitoneal route. This preliminary study indicated that plasmid(s) might be associated with virulence/pathogenicity in this species.

Gupta *et al.* (1996) opined that the invasiveness and lethality of *S. gallinarum* isolates were two different characteristics. Lethal organisms were highly invasive also but all highly invasive organisms were not lethal. High invasiveness could lead to persistence of organisms for longer time.

Joseph *et al.* (1999) analyzed the plasmid profile of 13 *S. gallinarum* isolates. Eleven isolates had both 85 and 2.5 kb plasmids. Isolate E-2455 had no

plasmid while isolate E-2450 had only 85 kb plasmid. Virulence study in one week-old chicks showed that only strains harbouring 85 kb plasmid were virulent. They could detect 472 bp plasmid associated virulence gene by PCR. Hence, it could be concluded that PCR detection of virulence gene, presence of 85 kb plasmid and virulence in one week-old chicks were all correlated and as such virulence could be measured by detecting the presence of virulence gene by PCR in place of plasmid profiling or animal inoculation studies.

Bhattacharya *et al.* (2000) conducted studies on amplification of virulence gene, presence of 90 kb plasmid and virulence in mice and chicks in *S. typhimurium* poultry isolates. By PCR a 472 bp product of virulence gene was amplified in all the 20 isolates of *S. typhimurium* tested. Two isolates which did not cause any mortality and were without any plasmids were also found to amplify virulence gene. The virulence study in mice and chicks showed difference in virulence of different isolates of *S. typhimurium* despite the presence of 90 kb plasmid and amplification of virulence gene. So, the intensity of virulence was not dependent only on the presence of 90 kb plasmid and amplification of virulence gene. The full expression of bacterial virulence depends upon the susceptibility of host and the presence of functional genes in the strain to complement the virulence.

Fierer and Guiney (2001) reported that variations of lipopolysaccharide (LPS) structure affected virulence of the strain since much of the antibody response to *Salmonella* infection was directed against the LPS. Other important virulence determinants of *Salmonella* were differential expression of various fimbriae, Salmonella Pathogenicity Island-1(SPI-1) which was needed to establish a separate niche in epithelial cells; SPI-2 helping *Salmonella* to manipulate the sorting of endosome or phagosome, altering the intracellular environment and facilitate bacterial growth within infected cells and SPI-3, helping bacteria to adapt to low Mg^{2+} and low pH of the endosome. *Salmonella* virulence plasmid (Spv plasmids) genes promoted the macrophage phase of

disease, dissemination from bowel and establishment of extra intestinal niches. Islets are small genetic loci or individual genes that encoded virulence traits and are variably present in different *Salmonella* lineages or individual strains. A gene called *shd* A was associated with faecal shedding, *sop* E gene with encoding an accessory epithelial cell invasion factor and *sod* C1 gene with encoding one of the two periplasmic superoxide dismutase enzymes.

Jones *et al.* (2001) studied the type III secretion systems encoded by SPI-1 and SPI-2 *in vitro* and the role of these pathogenicity islands in virulence in chickens. They found that SPI-2 function was required by *S. gallinarum* for virulence, primarily through promoting survival within macrophages, allowing multiplication within the reticuloendothelial system, which did not preclude the involvement of SPI-2 in uptake of organisms from the gut to the spleen and liver. Salmonella Pathogenicity Island-1 appeared to have little effect on virulence and survival of *S. gallinarum* in the host.

Audisio and Terzolo (2002) reported that mortality of the experimentally infected chicken with *S. gallinarum* might vary depending on the strain used and the genetic lines of chickens employed.

Kokosharov and Phetisova (2002) conducted experiments for finding out haemolysin and aerobactin production which were considered as virulence factors of *S. gallinarum*. They used 12 *S. gallinarum* strains isolated from internal organs of dead hens in different poultry farms. None of the tested *S. gallinarum* strains produced β-haemolysin but three of them (25.0 per cent) produced α-hemolysin. These strains were lethal to rabbits after Intravenous inoculation while non hemolysin producing strains weren't lethal. Five of the strains (41.7 per cent) were aerobactin positive. There was no coincidence between this biochemical parameter of different strains and their pathogenicity for experimental animals.

The virulence factors involved in *Salmonella* infections are numerous and complex. Some of them are toxins like endotoxin, enterotoxin and cytotoxin, contributing to adherence like surface polysaccharide O antigen, flagellar H antigen and fimbriae. Capsular Vi polysaccharide, invasion proteins encoded by *Salmonella* for surface adhesions, proteins neutralizing toxic oxygen products of macrophages, virulence plasmids, iron chelating proteins and siderophores could also contribute to virulence (Carter and Wise, 2004).

The LPS in the outer membrane is an important virulence determinant. Not only the lipid A component is toxic (endotoxin), but the length of the side chain in the O-repeat unit hinders the attachment of the membrane attack complex of the complement system to the outer membrane. Lipopolysaccharide binds to LPS-binding protein (a serum protein), which in turn transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells, triggering the release of proinflammatory cytokines (Hirsh *et al.*, 2004).

Muthuramalingam (2005) conducted a multiplex PCR on 15 *S. gallinarum* isolates. This procedure could simultaneously identify the *Salmonella* strains and the presence of virulence plasmid, thus facilitating the search for specific etiological agent. He could get the expected 571 bp level amplification, which was specific for *spv* virulence region and 284 bp level amplification, which was specific for genus *Salmonella* in all the isolates.

Shah *et al.* (2007) carried out a study to examine the role of *met* C gene in the virulence of *S. gallinarum* for chickens. They produced a *met* C (encoding cystathionine β lyase) mutant from a virulent strain of *S. gallinarum* by Mini-Tn5 insertional inactivation. The mutant was significantly attenuated in virulence for one-day-old White Leghorn chickens. Inactivation of *met* C resulted in 10^4-fold increase in the LD_{50} when compared with the wild type parent. The *met* C mutant showed an *in vivo* competitiveness defect in the challenged chickens and

significantly lower bacterial burden in the reticuloendothelial organs when compared with the wild-type parent. These results indicated that *met* C gene was important in virulence for *S. gallinarum* in chickens.

2.5 PATHOGENICITY OF *SALMONELLA GALLINARUM* IN NATURAL INFECTION

2.5.1 Pathogenicity in Naturally Infected Chicks

Mortality due to natural and experimental infection with *S. gallinarum* in chickens have been reported and varied from 0 to 100 per cent which could be attributed to wide variation in the virulence of different *S. gallinarum* strains (Singh *et al.*, 1996).

Rahman *et al.* (1997) reported an outbreak of salmonellosis among broiler chicks of one to six weeks of age in Assam, which caused an average mortality of 36 per cent within seven days. Two *Salmonella* serovars namely *S. gallinarum* and *S. indiana* were isolated from dead and ailing birds. The mortality was highest in chicks of age two weeks (46 per cent), then in four weeks (33.5 per cent) and least in six weeks of age (28.5 per cent).

Muir *et al.* (1998) compared *S. typhimurium* challenge model in 16 day-old chicken where the birds got infection from seeded litter by seeder birds infected orally. This trickle challenge exposed the susceptible birds to infection both orally, via the food, water and litter and percloacally. This situation more closely resembled the dynamics of field infection and should be used instead of single oral inoculations for assessment of vaccine efficacy. The distinguishing features of this mode of infection, compared with that following oral inoculation, were a slower rate of bird infection, a continual increase in colonization of cecum over a longer period of time (up to 15 days post challenge) and simultaneously, a slower establishment of systemic and mucosal antibody titers.

Newly hatched chicks were highly susceptible to oral infection with *Salmonella* during the first few days of life before their inhibitory microflora developed at three to six weeks of age (Virmani *et al.*, 2004).

2.5.2 Pathogenicity under Natural Infection in Layers

It is well known that *S. pullorum* and *S. gallinarum* are capable of chronically infecting ovary, resulting in eggs that are internally contaminated with salmonellae (Baker *et al.*, 1979).

Joshi *et al.* (2000) reported an outbreak of FT in a commercial layer farm with an overall strength of 18,000 layers and 6,000 grower chicken. *Salmonella gallinarum* was isolated from samples of liver, spleen and feces. The disease was observed only in layer flock of 60 weeks and above age groups and the birds of 80 week and 92 weeks of age were most affected. The overall mortality was 11.42 per cent, recorded in one week, with a maximum of 19.77 per cent in 80 weeks of age group, followed by 16.33 in 92 weeks and 13.07 per cent in 104 weeks of age. The clinical signs shown by affected birds were anorexia, ruffled feathers, huddling at one place, whitish diarrhea and in some cases articular swelling of hock or wing joints.

Rahman *et al.* (2002) reported that 4.76 per cent of layer birds maintained in a farm died due to natural outbreak of salmonellosis. *Salmonella typhimurium* could be isolated from liver, heart blood, spleen and intestinal contents. All the dead birds showed the same progression of clinical signs like general loss of condition, drowsiness, loss of appetite, ruffled feathers, leg weakness, whitish diarrhea, reduced egg production with shell less eggs/thin shelled eggs and loss of weight. Ova were misshapen, distorted, discolored and haemorrhagic.

2.6 PATHOGENICITY OF *SALMONELLA* IN EXPERIMENTAL INFECTION

2.6.1 Pathogenicity in Chicks After Experimental Infection

Holt and Porter Jr. (1991) infected white leghorn chicks of six weeks age with $1x10^9$ *S. enteritidis* and cloacal swabs were collected to study the release of *Salmonella* from intestine. The frequency of isolation of *S. enteritidis* was significantly higher in the secretions than in cloacal swabs by day nine post infection and by day 16 the *S. enteritidis* isolation rate was double that of cloacal swabs. This trend continued until the experiments ended on day 37. These secretions provided a much more sensitive sampling procedure than cloacal swabs, which were routinely used for studying the intestinal colonization or infection by enteric pathogens.

The pathogenicity of *S. pullorum* in northern bobwhite quail and mallard ducks were assessed and found that young bobwhites were susceptible to infection, with mortality and morbidity similar to those of chicks and poults. Antibody titres were detected one-week post infection (Buchholz and Fairbrother, 1992).

Krupeshsharma and Rajan (1996) produced experimental infection in three month-old Japanese quails using *S. typhimurium*. Within 24 h of infection, all quails became slightly dull and showed more fluid consistency of the fecal droppings. All the infected quails became dull and suffered progressive loss in weight till the eighth day as against the steady weight gain noticed in the control birds.

Rahman *et al.* (1997) injected $1x10^9$ bacteria/milliliter of *S. gallinarum* and *S. indiana* intraperitoneally to day-old chicks. They found that *S. indiana* caused 100 per cent mortality while *S. gallinarum* caused only 70 per cent mortality within a period of five days. Re-isolation of *Salmonella* from the heart

blood, liver and spleen of all dead birds confirmed that the outbreak was due to salmonellosis.

The chronic carrier state in *Salmonella* infection was associated with persistence of bacteria in the small intestine, spleen, and liver and chronic infection continued despite the development of protective immunity to challenge with virulent *Salmonella* (Sukupolvi *et al.*, 1997).

Gast and Holt (1998) experimentally infected day-old chicks with *S. enteritidis* and they found out that no livers or spleens were positive for organism after four weeks of age. But, *S. enteritidis* was found in at least 40 per cent of cecal samples cultured through 16 week of age.

Muir *et al.* (1998) reported experimental infection of *S. typhimurium* to 16 day-old chicks by two methods. One group of birds were challenged orally and another group through seeded litter. They found that birds challenged with *S. typhimurium* through litter experienced a more gradual infection compared with those receiving a single oral inoculum. All birds inoculated orally with *S. typhimurium* were shedding bacteria by day two post challenge (PC), but shedding in 100 per cent of litter challenged birds did not occur until day four PC. Subsequently, the orally challenged birds exhibited various or intermittent shedding frequencies of shedding by day six PC but all birds infected on seeded litter continued to shed *S. typhimurium* throughout the period of experiment.

Henderson *et al.* (1999) experimentally infected forty day-old chicken with *S. pullorum* at the rate of 10^8 CFU orally and could re-isolate salmonellae from cecum of challenged birds one day post inoculation. The number of cells then decreased to levels only detected by enrichment from second day PC.

Dhillon *et al.* (2001) conducted pathogenicity study in day-old chicks. The consistent gross and histopathological lesions were peritonitis, perihepatitis, yolk sac infection and enteritis. They reported that cecal pouches were the ideal organs for re-isolation of salmonellae at acute and chronic infection, compared with other organs. Mean body weights were reduced to 1.8 to 12.6 per cent in inoculated groups, compared to control groups. They suggested that tools such as body weight measurements, culture of organisms from cecal pouches, gross and histopathology should be undertaken to determine subclinical *Salmonella* infection in poultry.

Audisio and Terzolo (2002) analyzed the virulence of *S. gallinarum* strain by oral inoculation of 20-day-old chickens with a lyophilized culture of the bacteria. There was no correlation between the LD_{50} dose and the degree of disease produced. Disease started after an incubation period of five to six days.

2.6.2 Pathogenicity in Layers After Experimental Infection

Gast and Beard (1990) experimentally infected 20 to 22 week-old hens with *S. enteritidis*. The organism was recovered from internal organs of both orally inoculated hens and hens infected by horizontal contact transmission. *Salmonella enteritidis* was isolated from 58 per cent of ceca, 51 per cent of livers, 47 per cent of spleens and 17 per cent of the oviducts of hens sampled during the first five weeks after exposure. The organism was recovered at a low frequency from all internal organs sampled for as long as 22 weeks after exposure.

Shivaprasad *et al.* (1990) inoculated laying hens with *S. enteritidis* through various routes. They could isolate *Salmonella* organisms from eggshells, egg yolk and, albumen. Fecal shedding of organism was intermittent, which lasted for 18 days. The highest egg shell contamination was observed in intracloacally infected birds, while mean fecal shedding, drop in egg production and internal contamination of eggs were highest in intravenous inoculated group.

Nakamura *et al.* (1992) gave experimental infection of *S. enteritidis* to 34 week-old layers by three routes *viz.*, Oral, Intramuscular (IM) and Intravenous (IV). Within one week all IV group died and they found that egg production was significantly reduced in IM group during two to three weeks post inoculation. Out of 65 eggs examined from oral route of infection, 26 were shell positive, and one white positive whose shell was not contaminated. Out of 36 eggs examined from IM route of infection, 13 were shell positive, one yolk positive and two white and yolk positive without shell contamination. All birds inoculated by IV route died and *Salmonella* could be isolated from the oviduct.

Kokosharov *et al.* (1997) experimentally infected eight month-old New Hampshire layers orally with *S. gallinarum*. The clinical signs appeared first on fourth day with 25 per cent showing yellowish green droppings, some times admixed with blood and on the fifth day 75 per cent showed these signs. The feathers were ruffled, combs were shrunken and egg yield decreased.

Gast and Holt (1998) infected day-old chicks with *S. enteritidis* orally and during their 18 to 24 weeks of age 448 pools of egg contents (each containing up to three consecutive eggs laid by a single hen) were cultured for detecting contamination by *S. enteritidis* and only two egg pools were positive for *S. enteritidis*. Both of these pools were obtained from a single hen that was never observed to shed *S. enteritidis* in the feces.

Berchieri Jr *et al.* (2000) orally infected two commercial lines of layer chicken aged 44 and 46 weeks with *S. gallinarum*. They observed a drop in egg production for 12 days from the fifth day post infection (PI).

Holt *et al.* (2000) experimentally infected 35 week-old layers with *S. enteritidis* and observed that more than 90 per cent of fecal samples were culture positive at first week PI and by fourth week, only 20 per cent were culture positive.

All the 480 eggs laid within a period of 35 days by 48 eighteen week-old layers experimentally infected with *S. gallinarum* when examined bacteriologically were negative for salmonellae (Oliveira *et al.*, 2004).

2.6.3 Pathogenicity to Mice

Kokosharov and Phetisova (2002) tested pathogenicity of 12 *S. gallinarum* strains isolated from organs of dead hens from different poultry farms and they found that eight of them (66.7 per cent) were lethal for adult white mice after intraperitoneal inoculation.

2.7 LESIONS IN SALMONELLOSIS

2.7.1 Gross Lesions

Verma (1968) reported gross lesions produced by salmonellosis in laboratory animals like rabbits, hamsters, mice and guinea pigs due to *S. typhimurium* and *S. enteritidis*. He observed congestion of lungs, enlargement of liver and spleen with multiple necrotic foci. Intestine was highly congested with staining of intestinal contents.

Krupeshsharma and Rajan (1996) produced experimental infection in three month-old Japanese quails with *S. typhimurium*. The birds which died within 24 h of the treatment had moderate to severe congestion of the liver, kidney, spleen and heart. Two to three pin head sized grayish-white necrotic foci were seen on the liver. The birds which were sacrificed on 15th day had moderate hepatic congestion and few to many grayish white foci of necrosis scattered throughout the liver. Spleen and heart were moderately congested. There was catarrhal enteritis in all birds, but it was more severe in quails which died naturally.

Kokosharov *et al.* (1997) experimentally infected eight-month old New Hampshire layers orally with 18 h bacterial culture of *S. gallinarum*. It caused mortality in 50 per cent of experimental birds. Major lesions noticed were enlarged, friable and hyperemic liver, with distended gall bladder, spleenomegaly and catarrhal to haemorrhagic enteritis.

Day-old chicks were infected with *S. gallinarum* and *S. indiana* through intra-peritoneal route. The macroscopic alterations were severe to moderate congestion of intestinal tract, congestion and enlargement of liver and spleen. Pericarditis with occasional congestion and enlargement of kidney and lungs were also noticed (Rahman *et al.*, 1997).

Joshi *et al.* (2000) reported an outbreak of FT in a commercial layer farm in Madhya Pradesh and the gross lesions observed were enlargement with presence of grayish white nodules on heart, liver and gizzard. Few birds revealed hydropericardium and bronze discoloration of liver along with spleenomegaly. The ovaries were haemorrrhagic with misshapened and discolored ova. The intestines showed catarrhal inflammation and ulcers.

The main lesions observed on experimental oral infection with *S. gallinarum* were enlarged liver and spleen with necrotic and haemorrhagic foci (Berchieri Jr *et al.*, 2000).

Macroscopically, enlargement of liver and spleen with white necrotic foci were reported from 4^{th} and 8^{th} day respectively in *S. gallinarum* infection of day old chicks (Shah *et al.*, 2000).

In seven day-old chicks experimentally infected with *S. gallinarum* Prakash *et al.* (2002) observed enlarged bronze colored liver with necrotic foci,

congested heart with focal areas of haemorrhages, congested spleen and petechial haemorrhages in the intestine.

Rahman *et al.* (2002) reported an outbreak of salmonellosis due to *S. typhimurium* in a layer farm. All the birds which died of infection showed oophoritis with ruptured ova and salpingitis. Egg bound condition was observed in all cases. Discrete areas of necrosis were present on the walls of cecum and colon. Liver was slightly enlarged and congested. Spleen was moderately enlarged. Lungs and kidneys were also congested.

.2.7.2 Histopathological Lesions

Krupeshsharma and Rajan (1996) produced experimental infection in three month-old Japanese quails using *S. typhimurium*. The histologic changes were typical of bacterial hepatitis. There were focal to multifocal areas of coagulative necrosis of the liver and moderate fatty change. In focal areas, infiltration by heterophils was evident. Spleen showed reticular hyperplasia and moderate to severe congestion. Heart showed mild myocardial degeneration with engorged capillaries. In brain and kidney mild to moderate congestion was noticed. All infected birds had catarrhal enteritis.

Kokosharov *et al.* (1997) experimentally infected eight month-old New Hampshire layers orally with *S. gallinarum*. Histopathologically, liver revealed congestion, diffuse vacuolar degeneration and reactive necrosis. Myocardium showed hyperaemia, haemorrhage and lymphocytic infiltration. Lungs were hyperemic. Kidney showed congestion, parenchymatous degeneration and bacterial embolism.

Joshi *et al.* (2000) reported an outbreak of fowl typhoid in a commercial layer farm in Madhya Pradesh. The main observations were coagulative necrosis of hepatic cells and small necrotic foci in the parenchyma, with infiltration of

lymphocytes. The myocardium showed large areas of fibrinoid necrosis. Lesions of interstitial pneumonia with lymphocyte infiltration, few giant cells and RBCs were recorded in lungs. The proventriculus and intestines showed foci of necrosis. In uterus there was necrosis of smooth muscle fibers with presence of immature fibrinous tissue and infiltration of lymphocytes and heterophils. The ova contained erythrocytes and serous exudates in the central wall.

Prakash *et al.* (2002) reported congested vessels with multifocal necrotic areas infiltrated with heterophils and lymphoid cells in liver of experimentally infected chicks. Heart showed focal myocarditis characterized by congestion, hemorrhages, heterophil infiltration and oedema, resulting in separation of cardiac fibers and loss of cross striations. Mild lymphocytolytic activity of lymphoid component with increased haemosiderosis was seen in spleen. Multifocal areas of haemorrhage and moderate goblet cell hyperplasia were noticed in intestine.

Prasanna *et al.* (2001) produced experimental FT and Pullorum disease (PD) in one week-old chicks. In both FT and PD, the histopathological lesions in intestine were degeneration and desquamation of lining epithelial cells, haemorrhages, hyperactivity of the goblet cells and infiltration of heterophils and mononuclear cells in the lamina propria of the villi. Liver in both the diseases showed degeneration, early vacuolar changes in the hepatocytes, hypertrophy of Kupffer cells, focal infiltration of mononuclear cells and dilatation of hepatic sinusoids and necrosis.

2.8 DIAGNOSIS OF AVIAN SALMONELLOSIS

2.8.1 Isolation and Identification of Causative Agent

2.8.1.1 Isolation

2.8.1.1.1 Media

Selective enrichment on modified semisolid Rappaport-Vassiliadis (RV) agar was more reliable than Tetrathionate Brilliant Green (TBG) broth for isolation of *Salmonella* from samples of fecal origin (Poppe *et al.*, 1992).

Oboegbulem (1993) reported that RV medium when incubated at 43°C was significantly more efficient in recovering salmonellae from chicken carcass rinse fluids and Moore's sewer swabs.

Huang *et al.* (1999) reported two culture procedures for isolation of *Salmonella* in food. Bacteriological Analytical Manual (BAM) procedure included pre-enrichment in nutrient broth (NB) for 16 h, followed by selective enrichment in either RV or Tetrathionate Brilliant Green broth for 16 h. The Enrichment-Imuno Assay (EI) procedure included pre-enrichment in NB for 4 h, selective enrichment in RV for 16 h and post enrichment in NB for 4 h. They found that in the BAM procedure, the numbers of *Salmonella* in RV broth were more than that in Tetrathionate Brilliant Green broth for selective enrichment. They opined that RV might be a better selective enrichment for conventional differentiating agar because more *Salmonella* colonies would be present and less chance to be missed. When used for ELISA, Tetrathionate Brilliant Green broth showed more positive results than RV which might be due to reduced expression of antigenic epitope(s) in LPS detected by capture ELISA in RV medium.

Konwar and Joshi (2000) conducted a comparative study on the performance of five selective enrichment broths for the isolation of *Salmonella* from naturally contaminated raw meat of different animals. The media used were Muller-Kauffman tetrathionate broth at 43°C, RV medium at 42°C, selenite-

brilliant green broth at 37°C, selenite-cystine broth at 37°C and tetrathionate broth at 43°C. The best results were obtained with RV medium which detected 100 per cent of all positive samples, followed by Muller-Kauffman tetrathionate broth, tetrathionate broth, selenite-brilliant green broth and selenite-cystine broth. So they recommended RV medium incubated at 42°C for 24 h for selective enrichment of salmonellae. All other enrichments required 48 h of incubation for selective enrichment.

Shah *et al.* (2000) conducted studies to find out the comparative efficacy of different culture media like Mac Conkey Agar (MCA) and Brilliant Green Agar (BGA) as selective plating medium and tetrathionate broth as an enrichment medium for isolation of *S. gallinarum*. They found that MCA was superior to BGA as more number of isolations were possible on MCA. Tetrathionate broth, when used as an enrichment medium might have suppressed the growth of *S. gallinarum* and was not suitable for selective enrichment.

Audisio and Terzolo (2002) used novobiocin at the rate of 40µg/ml in enrichment medium for re-isolation of salmonellae from fecal samples. Novobiocin was added to prevent growth of *Proteus* spp., a frequent contaminant producing *Salmonella*-like colonies.

Gast *et al.* (2004) used an electrostatic air sampling device to collect *S. enteritidis* from rooms containing experimentally infected laying hens. Collection was done using electrostatic devices onto plates of six types of culture media *viz.*, BGA, modified Lysine iron agar, modified semisolid RV agar, Rambach agar, Xylose Lysine Deoxycholate (XLD) agar and Xylose Lysine Tergitol (XLT)-4 agar and were incubated at 37°C for 24 h. They found that frequency of positive air sampling results using BGA (66.7 per cent overall) was significantly greater than was obtained using most other media.

Musgrove *et al.* (2005) isolated salmonellae from egg rinses and crushed shells and membranes. Samples were pre-enriched in buffered peptone water at 35°C for 18 to 24 h, followed by enrichment in Tetrathionate broth and RV broth overnight at 42°C. Enriched samples were plated onto brilliant green sulfa and XLT-4 agar plates and incubated at 37°C for 18 to 24 h. Those samples with presumptive positives were inoculated into Lysine Iron Agar and Triple Sugar Iron (TSI) slants and incubated at 35°C for 18 to 24 h. Those samples with presumptive results indicative of *Salmonella* on these media were confirmed by serogrouping antisera.

2.8.1.2 Identification

2.8.1.2.1 Cultural characters

Salmonellae are aerobic and facultatively anaerobic. They grow well on laboratory media at 37°C without the addition of blood, serum, ascitic fluid, glucose, and after overnight incubation in broth a smooth strain will have produced an even turbidity, usually with no pellicle formation. With the majority of serotypes, growth on agar gives rise to round, smooth colonies of two to three millimeter diameter. However, a few serotypes, including *S. pullorum*, *S. choleraesuis* and *S. abortus ovis*, grow less abundantly on solid media and develop characteristically as small dew-drop like colonies after 18 h and even after 48 h incubation (Buxton and Fraser, 1977).

Salmonella gallinarum grew on blood agar as well defined, opaque, glistening colonies and would grow readily on MCA as pale, non lactose fermenting colonies and also on Deoxycholate-Citrate agar (DCA) and BGA. They could grow in selective enrichment media and of this selenite and tertathionate broth were most frequently used. *Salmonella gallinarum* and *S. pullorum* have many similar biochemical, cultural and serological properties, however *S. gallinarum* grew more readily on solid media and produced larger

colonies in contrast to *S. pullorum* which ferments maltose and dulcitol. Like *S. pullorum* this bacterium can survive outside the host's body for many months (Wray *et al.*, 1996).

2.8.1.2.2 Biochemical tests for Salmonellae

Most of the salmonellae do not ferment lactose, sucrose or salicin, and that they do ferment glucose, maltose, mannitol, dulcitol and dextrin with production of acid and gas. They are also characterized by their ability to reduce nitrates to nitrites and to produce H_2S. They do not decompose urea, liquefy gelatin or produce indole (Buxton and Fraser, 1977).

Nakamura *et al.* (1992) confirmed *Salmonella* biochemically by TSI agar, sulphide-indole motility (SIM) medium, lysine decarboxylase tests and serologically by plate agglutination test using antiserum prepared in rabbits.

Salmonellae are Gram-negative rods usually motile with a few exceptions. They are aerobic and facultatively anaerobic. They are catalase positive and oxidase negative, attack sugars by fermentation with production of gas. They usually give positive reactions for Simmons'citrate and lysine decarboxylase (Barrow and Feltham, 1993).

Salmonella gallinarum has the somatic structure 0, 1, 9, 12 and thus classified in group D of the Kauffman-White scheme. *Salmonella gallinarum* fermented glucose, mannitol, maltose and dulcitol but did not ferment lactose, sucrose and salicin (Wray *et al.*, 1996).

Shivhare *et al.* (2000) characterized the salmonellae isolates biochemically by tests like TSI agar, catalase, oxidase, ONPG, urease, methyl red, indole at 37°C, Voges Proskauer (VP), H_2S production, citrate utilization (Simmon's and Christensen's methods), nitrate reduction, phenyl-alanine

deamination, gelatin hydrolysis, decarboxylase (arginine, ornithine and lysine) tests and carbohydrate fermentation reactions using 16 sugars. Serological confirmation was done with the help of slide agglutination test using polyvalent 'O' and 'H' antisera.

Muthuramalingam, (2005) biochemically characterized 15 *S. gallinarum* isolates. All the isolates tested were indole negative, methyl red positive, Voges-Proskauer negative, urease negative, phenyl alanine deaminase negative, citrate negative and they were able to produce H_2S in TSI agar. Regarding the fermentation of sugars all isolates fermented dulcitol, maltose, arabinose, trehalose and mannitol. They could not utilize sucrose, cellobiose and lactose.

2.8.3 Serology

2.8.3.1 Agglutination tests

Bouzoubaa *et al.* (1987) used microantiglobulin test and microagglutination test to find out antibody titer in birds vaccinated against FT. They found that microantiglobulin test was much more sensitive than microagglutination test in detecting antibodies against *S. gallinarum*.

Waltman and Horne (1993) screened 1, 34,171 serum samples over a period of three years by the pullorum, typhoid tube agglutination test. Among the 680 reactors, *Salmonella* organisms were isolated from 226 of the chickens which included thirteen serotypes. The predominant serotypes were *heidelberg*, *pullorum*, *kentuck*, *saintpaul*, and *enteritidis* isolated at the rate of 60, 18, five, four and three per cent respectively. Highest number of isolations was obtained from ceca, followed by cecal tonsils and ovary-oviduct pool. The study reemphasized the tendency of pullorum and typhoid agglutination tests to give false positive reactions.

Gast (1997) conducted a study to evaluate the ability of Standard rapid whole-blood plate agglutination test and serum tube agglutination test antigens to detect infection in Single Comb White Leghorn hens inoculated with large oral doses of *S. pullorum* isolates. They were tested using both of the above tests before inoculation and, at six weekly intervals post inoculation. Infection of chickens with all six recent *S. pullorum* isolates were detected by both plate and tube tests in the experiment, although the frequency of positive results was consistently greater with tube test than with plate tests. Both tests detected infections of chickens with antigenically standard strains of *S. pullorum* significantly more frequent than infections with antigenically intermediate or variant strains. It was also noted that both plate and tube tests gave positive results in nearly all culture-positive hens, the plate test less often gave positive antibody results that could not be supported by positive organ culture results but tube test identified a substantial number of birds as seropositive, that were apparently not actively infected at the time of blood collection. This might be either due to persistent antibodies in hens that might have already cleared an earlier transient *S. pullorum* infection or inaccurate identification of uninfected hens as seropositive.

Joshi *et al.* (2000) reported an outbreak of FT in a commercial layer farm in Madhya Pradesh. The overall mortality was 11.42 per cent recorded in one week, with a maximum 19.77 per cent in 80 weeks of age group followed by 16.33 in 92 weeks and 13.07 per cent in 104 weeks old flocks. The *Salmonella pullorum-gallinarum* antibodies were positive in plate agglutination test and an antibody titer of 640, 1280, and >1280 were recorded in birds of 104, 92 and 80 weeks of age.

2.8.3.2 Agar Gel Precipitation/ Agar Gel Immunodiffusion Test (AGPT/AGID)

Glenn *et al.* (1967) reported individual results of sonic treatment on five different population levels of eight enteric organisms including salmonellae for the preparation of bacterial extracts prior to precipitin analyses.

Singh and Sharma (1985) found that *Salmonella bareilly (S. bareilly)* enterotoxin was immunogenic in rabbits. The antibodies produced were quite specific to the enterotoxic factor and antigenicity withstood heat treatment up to 90°C (30 min), but it was lost at 100°C (30 min). The enterotoxic activity of the toxin was neutralized by homologous antiserum in 1:40 dilution. Although antiserum was raised against the partially purified enterotoxin, the observations revealed that antibodies present in the antiserum were quite specific to enterotoxic factor, because the antiserum in AGPT reacted only with enterotoxic preparations of *S. bareilly* and *S. saintpaul*, but not with preparations obtained from non-enterotoxigenic strains of *S. bareilly* or enterotoxigenic strains of *E. coli* or *Y. enterocolitica*. These observations suggested that antiserum against enterotoxin, partially purified by salt precipitation with ammonium sulphate at 60 per cent saturation level and dialysis, could possibly be used for the detection of *Salmonella* enterotoxin *in vitro*.

Holt *et al.* (2000) made use of an AGPT test to detect antibodies to *S. enteritidis* deposited in egg yolks of infected hens. This method had the advantage of easy collection coupled with lower stress on birds during sampling, compared with serum. The AGP test could be an important tool for individuals using serological testing to monitor the *S. enteritidis* infection within their flocks or as a rapid screen for vaccine responses. The assay could also be used in tandem with other AGP tests to screen for the presence of multiple avian pathogens.

Singh and Sharma (2000) found out that all cytotoxigenic salmonellae produced *Salmonella* cytotoxins (SCTs) which could be exploited to develop *Salmonella* genus specific serodiagnostic test and its production in quantities enough to produce precipitin line in AGPT might help to confirm suspected *Salmonella* isolates even in primary isolation laboratories.

2.8.3.3 Enzyme Linked Immuno Sorbent Assay (ELISA)

Timoney *et al.* (1990) reported the use of an ELISA based on gm flagellin as an alternative to traditional pullorum disease test which lacked specificity and sensitivity in diagnosing *S. enteritidis* infection. The flagellin ELISA was more sensitive than the rapid slide test with pullorum antigen, regardless of whether the sera were drawn from experimental or naturally infected birds. More over, substantial numbers of sera giving weak positive reactions in the Pullorum test were strongly positive in ELISA, suggesting that antibody responses to H and O antigens were independent.

Berchieri Jr *et al.* (1995) used an indirect ELISA using soluble whole cell antigen to screen serum samples obtained from breeder and layer flocks, some of which had shown clinical or bacteriological evidence of infection with *S. gallinarum* or *S. pullorum*. They observed good correlation between *Salmonella* infection and the presence of serum samples showing high optical density (OD) values. They also performed slide agglutination test by mixing a drop of serum with a drop of bacterial suspension made by emulsifying a colony of *S. gallinarum* on agar in phosphate buffered saline, which showed agglutination within one minute of mixing. ELISA was found to be more specific than slide agglutination and could assist in eliminating false positive reactions.

Muir *et al.* (1998) used an indirect ELISA for antibody detection in the serum of experimentally *S. typhimurium* infected 16 day-old chicken. They used ELISA plates coated with one micro gram of *S. typhimurium* LPS.

Singh and Sharma (2000) conducted studies for detection of four types of SCTs namely SCT-I, II, III, IV using serological tests, *viz.*, Biken test, agglutination test, fluorescent antibody technique (FAT), ELISA, dot ELISA, AGPT, passive haemagglutination inhibition test (PHIT) and serum neutralization test (SNT). The tests were compared to each other and with Vero cytotoxicity assay for their sensitivity and specificity, using rabbit anticytotoxin serum raised against purified SCTs. None of the anticytotoxin sera reacted with heterologous purified cytotoxin in any of the tests. They found that ELISA was the most sensitive test capable to detect SCTs in nanogram amounts, followed by dot ELISA, PHIT and AGPT. Biken test, agglutination test and FAT were qualitative tests and could detect cytotoxin on intact *Salmonella*, but these tests failed to detect many cytotoxigenic strains, especially those which could not excrete cytotoxin extracellularly.

2.8.3.4 Molecular Methods

Christensen *et al.* (1994) characterized 14 Danish and five German isolates of *Salmonella enterica* serovar Gallinarum biovar gallinarum obtained from outbreaks in Denmark and Germany by phenotypic and genomic methods. The genomic methods included plasmid profiling of 85 kb virulence plasmid, plasmid restriction profiling, ribotyping and pulsed-field gel electrophoresis (PFGE) of genomic DNA. Except PFGE all phenotypic and genomic methods strongly indicated that Danish and German isolates were of the same class. The PFGE of genomic DNA digested with *Xba* I, however separated Danish and German isolates. This was confirmed when *Not* I was used to digest DNA. This enzyme also separated German isolates into two groups.

A PCR assay was developed for the identification of *Salmonella* infection in chickens and was compared with the conventional culture procedure. The pair of primers used were those directed at the *inv* A gene. As expected, a 284 bp

fragment DNA was amplified from extracted DNA of infected organs by PCR. The assay was more specific and sensitive when compared to culture methods (Tuchili *et al.*, 1995).

Jenikova *et al.* (2000) used two pairs of primers namely *inv, spv* in single PCR assay. The advantage of multiplex PCR was that it could simultaneously identify the *Salmonella* strains, which had a virulence plasmid, thus facilitating the search for specific etiologic *Salmonella* serovars.

Joseph and Singh (2000) conducted studies to evaluate the usefulness of restriction endonucleaase analysis of total DNA for differentiating field isolates of *S. gallinarum*. Digestion of genomic DNA from 13 *S. gallinarum* isolates from different outbreaks in poultry farms obtained from IVRI was done using four different restriction endonucleases, namely, *EcoR* I, *Xba* I, *Pst* I and *Hinf* I. The plasmid profile was different in isolates studied. One isolate did not carry any plasmid and another (E 2460) had only 85 kb plasmid, while remaining eleven isolates had both 85 and 2.5 kb plasmids. The isolates which had no plasmid and other isolate which had only 85 kb plasmid have shown unique chromosomal restriction patterns in comparison to other isolates which had both plasmids. This indicated that these isolates were derived from clones different from that of other isolates. The results clearly indicated that random fragments of genomic DNA were useful for fingerprinting isolates of *S. gallinarum*.

Kotetishvili *et al.* (2002) developed a multilocus sequence typing (MLST) based on the 16S RNA, *pdu* F, *gln* A, and *man* B genes for *Salmonella*, and its discriminatory ability was compared to those of PFGE and serotyping. Pulsed-field gel electrophorosis differentiated several strains undifferentiable by serotyping, the strains of several PFGE types were further differentiated by MLST, which suggested that the discriminatory ability of MLST for the typing of *Salmonella* was better than that of serotyping and/or PFGE typing.

Phage typing was used in epidemiological studies to identify isolates with specific characteristics such as multiple resistance to antibiotics and enhanced

virulence. Examples of important phage types were *Salmonella typhimurium* DT (definitive type) 104 which exhibited multiple resistance to antibiotics and *Salmonella enteritidis* PT (phage type) 4 which was found in poultry products and was a common cause of food poisoning in humans (Quinn *et al.*, 2002).

Skwark *et al.* (2004) reported that PCR was a very useful method to identify *Salmonella* strains and to determine their virulence factors by amplification of characteristic genetic markers. They concluded that sensitive and rapid PCR method might be used not only for identification of *Salmonella* strains and for determination of their virulence factors but also for routine microbiological diagnosis of food pathogens.

Shah *et al.* (2005) reported cloning and sequence analysis of *rfb* S gene to identify two polymorphic nucleotides, one at position 598 (*Salmonella gallinarum*-specific) and other at position 237 (*Salmonella pullorum*-specific). Based on *S. gallinarum*-specific nucleotide found at position 598, an allele-specific PCR method was developed for serotype-specific detection of *S. gallinarum.* This PCR method was able to discriminate pure cultures of *S. gallinarum* from *S. pullorum* and other *Salmonella* serotypes from serogroup D in less than three hours. Serotype-specific detection of *S. gallinarum* was possible in less than 24 h when the PCR was applied on the presumptive *Salmonella* colonies obtained after overnight incubation of selective media plates streaked with the clinical material from diseased chickens. This *rfb* S allele-specific amplification assay was specific, reproducible and less time consuming than the standard bacteriological methods used to detect *S. gallinarum* and could be an effective molecular tool for rapid definitive diagnosis of FT.

Vadivoo and Ramaswamy (2006) conducted PCR assay to isolate and identify *Salmonella* species rapidly in poultry products and environment. They compared the sensitivity of PCR with standard culture methods. A multiplex PCR assay was also done to detect *S. enteritidis* and *S. typhimurium*. Out of the 231 samples examined 12 were positive by culture method and 26 were positive by

PCR assay. All the samples that were *Salmonella* positive by culture method were also positive by the PCR, but 14 of the 26 PCR positive samples were negative by culture method. The specificity and accuracy of PCR assay was 100 per cent and 93.9 per cent respectively. In the 231 samples tested by the PCR, a 284 bp product was obtained for 26 samples. All the 26 isolates confirmed as *Salmonella* by PCR were used in multiplex PCR to identify the *Salmonella* serotype. An amplified product of 429 bp was noticed for all the 26 isolates of *Salmonella,* a 250 bp amplified fragment for *S. enteritidis* and 620 bp amplified product for *S. typhimurium*.

2.9 ANTIGENICITY OF *SALMONELLA GALLINARUM*

2.9.1 Factors Affecting Antigenicity

Bouzoubaa *et al.* (1989) found that *S. gallinarum* MSG1 field strain proteins were antigenic and they compared it with 9R live vaccine strain for their protection against experimental FT in chickens. Proteins from *S. gallinarum* gave better protection (95 per cent) compared to 9R live vaccine (60 per cent).

The cell wall of *Salmonella* is of typical gram-negative bacteria, composed of LPS, and protein. The antigenic composition of the polysaccharide portion of the LPS in part determines the serotype. The kind and number of sugars together with the linkage between them determine the antigenic determinants comprising the O-antigens of the particular isolate. The O-antigens, together with the antigenic determinants on the surface of the flagella (H-antigens), which are possessed by most salmonellae, help to define an isolate serologically. This classification scheme is called the Kauffman-White scheme (Hirsh *et al.*, 2004).

Reparaz *et al.* (2004) reported that *Salmonella* possess surface structures that could induce protective humoral and cellular immune responses following experimental infection in poultry and these components included LPS, *omps*,

fimbriae and flagellin. They performed immunoblotting with sera from naturally *S. enteritidis* infected laying hens on the heat extract (HE) of *S enteritidis* and observed the strongest response (higher number of hen reactants) was against porins (97.3 per cent), followed by *ompA* (64.7 per cent). Reaction was also seen against SEF 14 (50.0 per cent), SEF 21 (60.8 per cent), LPS (81.8 per cent) and flagellin (47.3 per cent). Cross reaction was noticed against LPS (86.7 per cent) and porins (66.7 per cent) when immunoblotting was performed with healthy bird sera. Cross reaction was not observed in case of flagellin and fimbriae, indicating the specificity of antibodies generated against these surface components during an infection by *S. enteritidis* in hens.

2.9.2 Detectection of Antigenicity

2.9.2.1 Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Weber and Osborn (1969) conducted SDS-PAGE on different proteins and showed that the method could be used with great confidence to determine the molecular weights of polypeptide chains for a wide variety of proteins.

Udhayakumar and Muthukkaruppan (1986) reported at least thirty polypeptide bands in OMP profile of *S. typhimurium* ranging from 12 kDa to 120 kDa, which also included some amount of contaminating LPS.

Prakash and Krishnappa (2002) conducted antigenic analysis of biofilm as well as free cells of *S. gallinarum* proteins by SDS-PAGE. They found repression of 89 kDa, 86 kDa and 34 kDa proteins in biofilm cells compared to free cells. At the same time 66 kDa, 59 kDa, 57 kDa, 45 kDa, and 38 kDa proteins were over expressed in biofilm cells.

Sujatha *et al.* (2003) characterized *S. gallinarum* antigens from field isolates using SDS-PAGE and their results revealed that molecular weight of antigenic moieties of the isolates ranged from 14 kDa to 97 kDa. The number of bands obtained ranged from 19 to 26.

Lee *et al.* (2005) conducted experiments with seven epidemiologically unrelated *S. gallinarum* strains. The crude protein extract of all these isolates when subjected to SDS-PAGE showed migration of *omp*s as several bands at approximate molecular weights of 99 kDa, 87 kDa, 77 kDa, 63 kDa, 56 kDa, 54 kDa, 51 kDa, 49 kDa, 47 kDa, 45 kDa, 43 kDa, 41 kDa, 39 kDa, 37 kDa and 32 kDa. The 39 kDa *Omp* C protein revealed by all strains of *S. gallinarum* might be an effective inducer of immune response, since Western immunoblot analysis with anti-*Omp* C rabbit serum and serum from infected chicken showed reaction to characteristic 39 kDa band.

Rathore *et al.* (2005) had studied the protein profile of sonicated extract antigen of two *S. gallinarum* isolates by SDS-PAGE. They found that one strain, E-76 showed approximately 32 bands with 14 major bands, while other strain, E-3019 showed 27 bands with seven major bands.

Materials and Methods

3. MATERIALS AND METHODS

Glassware of Borosil brand and Tarsons and Genei brand plastic ware were used in this study. All chemicals used were of Molecular biology grade, obtained from Bangalore Genei and Sisco Research Laboratories Private Limited (SRL). Ready-made media were procured from Hi-Media Laboratories Private Limited, Mumbai, unless otherwise mentioned.

3.1 BACTERIAL ISOLATES

Four *S. gallinarum* isolates were used for the present study *viz.*, BGL, PS 8, QS 1 and X 4. The isolate BGL was considered as reference strain and was kindly provided by Dr M. Sathyanarayana Rao, Professor and Head, Department of Veterinary Microbiology, Veterinary College, Bangalore. The isolates PS 8 and X 4 were from chicken while QS 1 was isolated from quail. These three isolates were obtained from ailing/dead birds brought to the Department of Microbiology/ Pathology, College of Veterinary and Animal Sciences (COVAS), Mannuthy for detailed disease investigation. By standard bacteriological procedures and molecular techniques like genus specific and serovar specific PCR, isolates PS 8 and QS 1 were identified as *S. gallinarum*. The isolate X 4 was serotyped as *S. gallinarum* at Indian Veterinary Research Institute (IVRI), Izatnagar. All the isolates were maintained in the Department of Microbiology, COVAS, Mannuthy.

3.2 STORAGE OF ISOLATES

3.2.1 Materials

3.2.1a Glycerol

Glycerol was sterilized using hot air oven at 160°C for one hour.

3.2.1b Brain Heart Infusion Broth (BHIB)

Obtained as dehydrated powder and was reconstituted as per manufacturer's instructions.

3.2.1c *S. gallinarum* Isolates

Four isolates *viz.*, BGL, PS 8, QS 1 and X 4.

3.2.2 Method

A single colony of each *S. gallinarum* isolate was added to 4.5 millilitres of BHIB and incubated at 37°C for 18 to 24 h. After incubation, to each of the test tubes 0.5 milliliters of sterile glycerol was added and mixed well. The test tubes were then labeled and stored at 4°C. The isolates were revived once a month.

3.3 REVIVAL OF ISOLATES

3.3.1 Materials

3.3.1a *S. gallinarum* isolates in glycerol broth

3.3.1b Mac Conkey Agar/Brilliant Green Agar (MCA/BGA)

Obtained from Hi-media and was used as per manufacturer's instruction.

3.3.2 Method

The isolates were revived once a month. The stored glycerol broth was thawed to room temperature (RT) and was mixed well. Then a loop full was plated on MCA/BGA and incubated at 37°C overnight. Pale yellow colonies on MCA or pink colonies on BGA were selected and further purity was checked by standard bacteriological procedures.

3.4 ANTIBIOGRAM

3.4.1 Materials

Mueller-Hinton agar was used to study the antibiotic sensitivity pattern of the isolates. The following antibiotic discs with known concentrations as noted

in micrograms (µg) or international units (IU) per disc were used (Hi-Media Laboratories Private Limited, Mumbai, India).

1. Amoxy clav (Ac) – 10 µg
2. Cloxacillin (Cx) - 1 µg
3. Penicillin G (P) - 10 units
4. Enrofloxacin (Ex) - 10 µg
5. Nalidixic acid (Na) – 30 µg
6. Gentamicin (G) - 30 µg
7. Tetracycline (T) - 10 µg
8. Erythromycin (E) - 15 µg
9. Chloramphenicol (C) - 3 µg
10. Co-trimoxazole (Co) - 25 µg
11. Clindamycin (Cd) - 2 µg
12. Ciprofloxacin (Cf) - 10 µg
13. Cephalexin (Cp) - 10 µg

3.4.2 Method

Antibiotic sensitivity test was done as per the standard single disc diffusion method of Bauer *et al.* (1966).

3.5 PATHOGENICITY TESTING OF ISOLATES

3.5.1Pathogenicity to mice

3.5.1.1 Materials

3.5.1.1a Swiss Albino Mice

Swiss Albino mice, six to eight weeks of age were procured from the Small Animal Breeding Station (SABS), COVAS, Mannuthy.

3.5.1.1b S. gallinarum isolates

3.5.1.2 Method

Each mouse was inoculated intra-peritoneally with 0.2 milliliter of inoculum containing 3 x 10^8 organisms per ml in sterile normal saline. Three mice were used for each isolate. One mouse per isolate was used as control and these animals received one milliliter of sterile normal saline intra-peritoneally. The inoculated animals were observed for clinical symptoms/death for a period of seven days. From the dead animals, attempts were made to re-isolate *S. gallinarum* from heart blood, lung, liver, spleen and ceca.

3.5.2 Pathogenicity Testing in Day-old Chicks

3.5.2.1 Materials

3.5.2.1a Day-old chicks

Ninety-six, day old *Salmonella* free Grama Priya breed of chicks of either sex vaccinated against New Castle disease (ND) were procured from Centre for Advanced Studies in Poultry Science, COVAS, Mannuthy. The chicks were divided into 12 test groups, each containing five chicks and 12 control groups, each containing three chicks. Three test groups and three control groups were used per isolate for determining the pathogenicity through three different routes of inoculation. Test group for BGL isolate was assigned as Group I, for PS 8 as Group II, for QS 1 as Group III and for X 4 as Group IV. The suffix 'a' was given for all oral test groups, 'b' for intra muscular and 'c' for intra cloacal routes. During the first week chicks were given 24 hours of artificial light and there after 12 hours at night. Birds were fed with chick mash and watered *ad libitum.*

3.5.2.1b BHIB

3.5.2.1c Tripticase Soya Agar (TSA)

3.5.2.1d Revived S. gallinarum isolates (3.3)

3.5.2.1e Phosphate buffered saline (PBS) pH 7.2

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate dihydrate	1.06g
Potassium dihydrogen phosphate	0.2 g
Distilled water to	1000 ml

pH adjusted to 7.2 and autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.5.2.2 Method

3.5.2.2.1 Preparation of infective inoculum

The infective inoculum was prepared as described by Dhillon *et al.* (2001), with suitable modifications. A pure single colony of each of the four isolates was inoculated into five milliliters of BHIB and incubated for six hours at 37°C. This was seeded on TSA plates and was incubated overnight. The bacteria were harvested using sterile PBS and was washed thrice with PBS by centrifugation at 3000 x g for 30 min. Then the concentration was adjusted to obtain the inoculum having approximately 3 x 10^8 bacteria/ml as per Me Farland's standard tube number one and stored at 4°C till use.

3.5.2.2.2 Experimental infection through oral route

Inoculum at the rate of 0.5 milliliters was given to each chick by directly inserting a tuberculin syringe into the crop. Equal amount of sterile PBS was given to control birds in the same way.

3.5.2.2.3 Experimental infection through Intra muscular (IM) route

The test group chicks were given 0.5 milliliters of inoculum by IM route at breast/thigh muscle. Control groups were given 0.5 milliliters of sterile PBS in the same manner.

3.5.2.2.4 Experimental infection through Intra cloacal (IC) route

Three test groups and three control groups were selected for each of the four isolates and 0.5 milliliters of the inoculum was swabbed into the cloaca of

each test group chicks using a sterile cotton swab. Control groups were also treated similarly. Instead of inoculum sterile PBS was used.

All the chicks were observed for any clinical signs/mortality for a period of one month. Pooled fecal swab samples were collected from each group on days 3, 7, 11, 15, 21, 28 post inoculation (PI) and were subjected to microbiological examination for salmonellae.

Organ samples from dead chicks (ceca, spleen, heart, lungs and liver) were collected and examined for re-isolation of salmonellae.

Those birds, which survived after experimental period of 30 days, were checked for their body weight. These birds were sacrificed and tissue samples were examined for re-isolation of salmonellae.

Representative samples of tissues like liver, spleen, lungs, heart, ceca, ovary and oviduct were collected in 10 per cent neutral buffered formalin for histopathological examination.

3.6.3 Pathogenicity Testing in 18 Week Old Layers

Pathogenicity studies in layers was done as per Shivaprasad *et al.* (1990) with suitable modifications.

3.6.3.1 Materials

3.6.3.1a Layer birds

Seventy two, 18 week-old layers belonging to Indian White leg Horn strain- P (IWP) vaccinated against Marek's disease, ND, Infectious Bursal Disease (IBD) and Fowl Pox were procured from All India Co-ordinated Research Program (AICRP) on poultry, COVAS, Mannuthy. The *Salmonella* free status of birds were confirmed by Slide Agglutination Test (SAT). They were divided into 12 test groups, each with 4 birds and 12 control groups, each with 2 birds. Three test groups and three control groups were used per isolate for determining the pathogenicity through three different routes of inoculation. Test Group for BGL isolate was assigned as Group V, for PS 8 as Group VI, for QS 1

as Group VII and for X 4 as Group VIII. The suffix 'a' was given for all oral test groups, 'b' for intra venous and 'c' for intra cloacal routes. The birds were fed with layer mash and watered *ad libitum*. The birds were dewormed and a pre-experimental period of two weeks was given for adaptation and optimization of egg production.

3.6.3.1b BHIB

3.6.2.1c TSA (3.5.2.1c)

3.6.2.1d Revived S. gallinarum isolates (3.3)

3.6.2.1e PBS (3.5.2.1e)

3.6.3.2 Method

3.6.3.2.1 Preperation of infective inoculum

A single colony of each of the four isolates was inoculated into five milliliters of BHIB and was incubated for six hours at 37°C. This was further streaked on TSA plates and was incubated at 37°C for 18h. Bacteria were harvested in sterile PBS and washed thrice by centrifugation at 3000 x g for 30 min. Then the concentration was adjusted to obtain the inoculum having approximately 3 x 10^8 bacteria/ml as per Mc Farland's standard tube number one and stored at 4°C till use.

3.6.3.2.2 Experimental infection through oral route

One milliliter of inoculum was given to each bird in the test groups by directly inserting a blunt ended catheter attached to a tuberculin syringe into the crop. One milliliter of sterile PBS was given to control birds in the same way. The birds were deprived of feed and water for minimum of three hours before and after inoculation.

3.6.3.2.3 Experimental infection through Intra venous (IV) route

One milliliter of the inoculum was given to each of the test group bird by IV route at wing/tarsal vein. Control group was given one milliliter of sterile PBS in the same way.

3.6.3.2.4 Experimental infection through Intra cloacal (IC) route

One milliliter of the inoculum was swabbed into the cloaca of each test group bird using a sterile cotton swab. Instead of inoculum, cloaca of control birds were swabbed with equal quantity of sterile PBS.

Layers were observed for any clinical signs/mortality for a period of one month. Individual cloacal swabs were collected from each bird on days 3, 7, 11, 15, 21, 28 post inoculation (PI) and were subjected to detailed microbiological examination for salmonellae.

Organ samples from dead birds (ceca, spleen, liver, ovary and oviduct) were collected and subjected to re-isolation of *Salmonella.*

Pattern of egg production was noticed daily. The eggs were pooled per route per isolate and attempts were made to isolate *Salmonella.*

Those birds, which survived after experimental period of 30 days, were sacrificed and tissue samples were utilized for isolation of *Salmonella.*

Representative samples of tissues like liver, spleen, lungs, heart, ceca, ovary and oviduct were collected in 10 per cent neutral buffered formalin for histopathological examination.

3.7 RE-ISOLATION OF *SALMONELLA* FROM CLINICAL SAMPLES OF EXPERIMENTALLY INFECTED BIRDS

Re-isolation was tried from cloacal swabs, eggs, liver, spleen, heart, lungs, ovary, oviduct and ceca of experimentally infected birds.

3.7.1 Materials

3.7.1a Cloacal swabs and tissues from experimental birds

3.7.1b Eggs from experimental layer birds

3.7.1c Non-selective Pre-enrichment medium

Buffered peptone water (BPW) was prepared as per manufacturer's instruction and antibiotic novobiocin was incorporated at the rate of 0.5 microgram/milliliter.

3.7.1d Selective enrichment medium

Rappaport Vassiliadis soya broth (RVSB) was used as per manufacturer's instruction.

3.7.1e Selective Media for isolation

Brilliant Green Agar and MCA, were obtained as dehydrated media from Hi-Media Laboratories, Mumbai, India. The required media were rehydrated and prepared as per the instructions of the manufacturer and were supplemented with sulpha selective supplement at the rate of one micro liter/milliliter.

3.7.2 Method

3.7.2.1 Cloacal swabs

Cloacal swabs were directly collected into BPW and were incubated at 37°C for 24 h.

3.7.2.2 Organ Samples Collected After Post Mortem

Organ samples were triturated with minimum quantity of PBS using sterile mortar and pestle. About one milliliter of the above was added to BPW and incubated at 37°C for one day.

3.7.2.3 Eggs

Eggs from each experimental group were pooled and collected daily in sterile polythene bags. They were stored at 4°C for two to five days before attempting isolation of bacteria. They were broken by agitation and were incubated at 42°C overnight. A sterile cotton swab was used to collect the egg sample and this was inoculated in BPW and was incubated at 37°C for one day.

From the above inoculated, incubated BPW one-milliliter was taken, inoculated in to RVSB and further incubated at 42°C for 48 h. From this, direct plating was done on to selective media like MCA and BGA supplemented with sulpha. Gram-negative non-lactose fermenting colonies presented on MCA and pink colonies obtained on BGA were selected, and sub cultured to obtain pure culture for further identification.

3.8 IDENTIFICATION

The bacterial isolates were identified based on Gram's staining, cultural characteristics, tests for catalase and oxidase, indole production, methyl red and Voges-Proskauer reactions, urease activity, phenyl alanine deaminase, H_2S production, nitrate reduction, citrate utilization, ornithine decarboxylase activity as described by Barrow and Feltham (1993).

3.9 HISTOPATHOLOGY

Representative samples of tissues from liver, spleen, heart, lungs, ovary, oviduct and ceca of dead/sacrificed birds were collected and preserved in 10 per cent Neutral Buffered Formalin. The tissues were processed by routine paraffin embedding techniques (Sheehan and Hrapchak, 1980). Sections were cut at four micron thickness and stained with routine Haematoxylin and Eosin stain (Bancroft and Cook, 1995) for histopathological studies. The stained sections were subjected to detailed examination under the light microscope and the lesions were classified.

3.10 ANALYSIS OF OUTER MEMBRANE PROTEINS (OMPS) OF *S. GALLINARUM*

3.10.1 Extraction of OMP

3.10.1.1 Materials

3.10.1.1a PBS (3.5.2.1e)

3.10.1.1b Tris-HCl 20 mM (pH 7.2)

Tris-HCl	3.15 g
Distilled water to	1000 ml

pH adjusted to 7.2 , autoclaved and stored at 4°C.

3.10.1.1c HEPES (N-2-hydroxyethyl Piperazine N-2 ethane sulphonic acid) buffer, 10mM , pH 7.4

HEPES buffer	0.238 g
Distilled water to	100 ml

pH was adjusted to 7.4, autoclaved and stored at 4°C.

3.10.1.1d Sodium N-lauroyl sarcosine (0.5 per cent) (Sarkosyl,Sigma Aldrich, USA

500 mg of sodium lauroyl sarcosinate was dissolved in 100 ml of sterile 10 mM HEPES buffer (pH 7.4) just prior to use.

3.10.1.1e BHIB

3.10.1.1f S. gallinarum isolates in glycerol broth

3.10.1.2 Method

The outer membrane protein (OMP) enriched extract from *S. gallinarum* was prepared as per the method described by Davies and Donachie (1996).

A single colony of each of the four isolates (BGL, PS 8, QS 1 and X 4) were inoculated into 10 ml of sterile BHI broth and incubated over night at 37°C.

Two-liter flask containing 400 ml of BHI broth was pre-warmed overnight at 37°C. Overnight grown culture, approximately 0.4 ml was added to 400 ml of pre-warmed medium and incubated for 12 h at 37°C. The cultures were placed on ice to stop the bacterial growth.

The bacteria were harvested by centrifuging at 8000 x g for 30 min at 4°C. The bacterial pellet was washed twice in sterile PBS (pH 7.2) and the final pellet was dissolved in seven milliliters of ice-cold HEPES buffer (pH 7.4) and kept for 20 min. Bacterial cells were sonicated on ice for five minutes (Branson Sonifier 450) at 12 microns by placing the probe into the suspension to a depth of 1.0-1.5 cm to avoid frothing. The sonicated sample was poured into a 15 ml centrifuge tube, placed on ice for 15 min and centrifuged at 4000 x g for 20 min at 4°C to remove the intact cells and debris. The supernatant was carefully transferred to 10 ml polyallomer ultracentrifuge tubes and centrifuged at 1,00,000 x g for one hour at 4°C in a Sorvall ultracentrifuge to pellet the cell envelopes.

The supernatant was discarded and the pellet was resuspended in seven millilitres of 0.5 per cent sodium N-lauroyl sarcosine and kept at room temperature for 20 min. The sarkosyl insoluble outer membrane enriched fraction was pelleted by centrifugation at 1, 00,000 x g for one hour at 4°C. The pellet was washed twice in distilled water and finally dissolved in 0.5 ml of Tris-HCl 20 mM pH 7.2 and was stored at -70°C.

3.10.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.10.2.1 Materials

3.10.2.1.a Acrylamide-bisacrylamide stock (30 : 0.8)

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Distilled water to make	100 ml

Filtered through Whatman No.1 filter paper and stored at 4°C.

3.10.2.1.b 1.5 M Tris pH 8.8

Tris base	181.7 g.
Distilled water to	1000 ml

Adjusted pH to 8.8 with 4N HCl. and stored at 4°C.

3.10.2.1.c 0.5 M Tris pH 6.8

Tris base	60.6 g.
Distilled water to	1000 ml

Adjusted pH to 6.8 with 4 N HCl. and stored at 4°C.

3.10.2.1.d Resolving gel (12.5 per cent)

Acrylamide: bisacrylamide (30: 0.8)	12.50 ml
Tris hydrochloride (1.5 M) pH 8.8	7.50 ml
Sodium dodecyl sulphate (ten per cent)	0.30 ml
Ammonium persulphate (ten per cent)	0.15 ml
N, N, N, N-tetra methyl ethylenediamine (TEMED)	0.01 ml
Distilled water	9.60 ml

3.10.2.1.e Stacking gel (four per cent)

Acrylamide stock (30.8 per cent)	0.67 ml
Tris hydrochloride (0.5 M) pH 6.8	1.25 ml
Sodium dodecyl sulphate (ten per cent)	0.05 ml
Ammonium persulphate (ten per cent)	25 µl
N, N, N, N - tetra methyl ethylenediamine (TEMED)	2.50 µl
Distilled water	3.00 ml

3.10.2.1.f Electrophoresis buffer

Tris base	3.0 g
Glycine	14.4 g
Sodium dodecyl sulphate	1.0 g
Distilled water to make	1000 ml

3.10.2.1.g Sample preparation buffer (2X)

0.5 M Tris hydrochloride, pH 6.8	2.5 ml
Glycerol	2.0 ml

Sodium dodecyl sulphate (ten per cent)	4.0 ml
2-mercaptoethanol	0.2 ml
Bromophenol blue	0.5 mg
Distilled water to make	10.0 ml

Distributed in small aliquots and stored at 4°C.

3.10.2.1.h Destaining solution I

Glacial acetic acid	70 ml
Methanol	400 ml
Distilled water to	1000 ml

3.10.2.1i Destaining solution II

Glacial acetic acid	70 ml
Methanol	50 ml
Distilled water to	1000 ml

***3.10.2.1j* Coomassie brilliant blue (SRL) staining solution**

Coomassie brilliant blue (R250)	0.5 g
Methanol	800 ml
Glacial acetic acid	140 ml
Distilled water to	2000 ml

3.10.2.2 Method

The OMPs of all the four *S. gallinarum* isolates were analysed by discontinuous system of polyacrylamide gel electrophoresis (Laemmli, 1970)

Resolving gel solution, 12.5 per cent was prepared and degassed. Ten per cent ammonium persulphate and TEMED were added and poured between two glass plates so as to form a gel of one millimeter thickness and left for polymerization. Distilled water was layered on the top to ensure uniformity of the gel surface. The set up was left overnight at 4°C for complete polymerization. After polymerization the distilled water was pipetted out and four per cent stacking gel was prepared and poured between the glass plates. The comb was then inserted and the apparatus left as such for complete

polymerization for one and half hours. After polymerization, the comb was removed and the wells were washed thoroughly with running buffer. A small quantity of running buffer was added into each of these wells. Twenty micro liters of the sample was mixed with equal volume of sample preparation buffer and kept in boiling water bath for ten minutes. The samples were loaded into individual wells under the column of buffer in each well. Standard Medium range protein molecular weight marker (GENEI) was loaded in one of the wells. The glass plates were fixed onto the vertical slab gel electrophoresis apparatus and electrophoresis buffer was carefully poured into the top and bottom reservoirs and electrophorosed at 10 mA constant current till the bromophenol blue marker reached near the bottom of the resolving gel. The gel was then removed from the glass plate; the stacking gel was snipped off and transferred to a Petri plate containing Coomassie brilliant blue staining solution for two to three hours. The gels were then destained till the background became clear and were photographed using Biorad gel documentation system.

3.11 ANALYSIS OF HOMOGENEITY/HETEROGENEITY OF BACTERIAL ISOLATES BY SEROLOGY

3.11.1 Preparation of Whole Cell Proteins as antigen

3.11.1.1 Materials

3.11.1.1a Antigen preparation buffer

Tris (0.1M)	1.200 g
Phenyl methyl sulphonyl fluoride	0.034 g
Glycerol	15.00 ml
SDS	2.000 g

pH adjusted to 6.8 and the volume made up to 100 ml with distilled water.

3.11.1.1b Four S. gallinarum isolates in glycerol broth

3.11.1.1c TSA (3.5.2.1c)

3.11.1.1d PBS (3.5.2.1e)

3.11.1.2 Method

Whole cell proteins were prepared as per the method of Ireland *et al.* (1991). Pure cultures of *S. gallinarum* isolates (BGL, PS 8, QS 1 and X 4) grown in TSA plates at 37°C for 18 h were harvested each in 10 milliliters of PBS. The cell suspension was adjusted to a concentration of $3x10^9$ cells/milliliter. The cell suspensions were washed thrice in PBS and was finally pelleted by centrifuging at 3000 x g for 30 minutes. Each of these pellets was again resuspended in 10 ml of antigen preparation buffer. The cell suspensions were mixed, placed in a boiling water bath for five minutes, centrifuged at 10,000 x g for three minutes and the supernatant collected. The protein concentration of the preparations were determined by the method of Lowry *et al.* (1951) using a bovine serum albumin (BSA) standard.

3.11.2 Raising of Antiserum against *S. gallinarum* Whole Cell Proteins

3.11.2.1 Materials

3.11.2.1a Whole cell protein extract from isolates BGL and QS 1 (3.11.1)

3.11.2.1b PBS pH 7.2 (3.5.2.1e)

3.11.2.1c Freund's complete and incomplete adjuvants

3.11.2.1d Rabbits

Young adult female rabbits (New Zealand White) weighing about one kilogram were obtained from SABS, COVAS, Mannuthy.

3.11.2.2 Method

Antiserum production was carried out as per Antony (2004).

3.11.2.1a Sterility test

The sterility of the whole cell protein suspension was checked by streaking 0.2 ml of the suspension on BHI agar, Blood agar, Sabouraud's Dextrose Agar (SDA) and inoculating in Thyoglycollate broth and incubating at 37°C for seven days.

3.11.2.1b Inoculation of rabbits

The sterility tested whole cell protein suspensions were thoroughly emulsified with equal volume of Freund's complete adjuvant. Two millilitre of each emulsified suspension (BGL) having whole cell protein concentration of 6.46mg/ml and QS 1 having whole cell protein concentration of 5.945mg/ml was inoculated by deep IM route to two rabbits.

A booster dose was given on day 14, followed by another dose on day 21. The antigens used for booster doses were emulsified with Freund's incomplete adjuvant.

3.11.2.1c Test bleedings

On 28th day the rabbits were bled from the ear vein, the serum separated and tested for presence of specific antibody by agar gel precipitation test.

3.11.3 Agar Gel Precipitation Test

3.11.3.1 Materials

3.11.3.1a Pre-coated slides

Clean glass slides were coated by smearing 0.5 per cent melted agar in distilled water and drying in air by keeping the slides horizontally over glass rods.

3.11.3.1b Gel for AGPT

Agarose	1.00 g
Sodium chloride	0.85 g
Sodium azide	0.01 g
Distilled water to	100 ml

To dissolve the agarose in saline, the solution was boiled for five minutes.

3.11.3.1c Whole cell protein antigen of four isolates (3.11)

3.11.3.1d Hyperimmune serum against S. gallinarum BGL and QS 1 isolates (3.11.2)

3.11.3.2 Method

Agar gel precipitation test was done as per the method of Pati *et al.* (1996) with minor modifications. Four milliliters of melted agarose was poured onto pre-coated glass slides and allowed to set. One central well and five peripheral wells, each with three millimeter diameter were punched in such a way that the distance between the central well and any peripheral well was three millimeters. Distance between the adjacent peripheral wells was kept equal. Two slides were prepared, one for isolate BGL and other for isolate QS 1. The central well was filled with 20 µl hyper immune serum. Each of the peripheral wells was filled with 20 µl whole cell protein from the 4 isolates and last well was filled with PBS to act as negative control. The slides were incubated at room temperature in a humid chamber for 24 h and were examined in diffuse light for the presence of precipitin lines and were photographed.

Results

4. RESULTS

4.1 REVIVAL OF ISOLATES

The *S. gallinarum* isolates, stored at 4° C in glycerol broth, were revived once in a month. All the isolates could be revived in MCA/BGA and were found to be pure.

4.2 ANTIBIOTIC SENSITIVITY TEST

Antibiogram of *S. gallinarum* isolates indicating the susceptibility and resistance to various antibiotics/antibacterial agents is presented in Table 1.

All the isolates were sensitive to gentamicin, chloramphenicol, enrofloxacin, tetracycline, amoxy clav, cephalexin and ciprofloxacin.

All *S. gallinarum* isolates were resistant to penicillin-G, nalidixic acid, clindamycin, cloxacillin and erythromycin. Except isolate X 4, all were found resistant to co-trimoxazole.

4.3 PATHOGENICITY

4.3.1 Pathogenicity in Mice

All the three local isolates of *S. gallinarum* were able to kill weaned mice, while the reference strain BGL didn't kill the mice even after seven days. Isolate PS 8 killed mice within 24 h, while isolates QS 1 and X 4 took 72 and 96 h respectively for causing mortality.

The gross lesions observed in the internal organs of the dead mice were petechiae of the pericardium and congestion of lung. Liver and spleen showed enlargement, with congestion and focal areas of necrosis. Re-isolation of *S. gallinarum* was possible from the heart blood, lungs, liver and spleen on MCA/BGA.

4.3.2 Pathogenicity in Day-Old Chicks

The results of pathogenicity studies by four isolates of *S. gallinarum* in day-old chicks are summarized in Table 3. The clinical signs and mortality were prominent and appeared early in IM route, followed by oral and IC route inoculated chicks. The major clinical signs observed were somnolence, poor growth, weakness, inappetence and whitish diarrhea. Laboured breathing and gasping were shown by chicks that died within 48 h after inoculation.

The chicks in Group Ia and Ic survived the experimental period. All the birds in Group Ib were found dead. One bird each died on first and second day. On the third day another two birds died, and one died on fifth day. Organisms could be re-isolated from the liver, spleen, heart blood, lungs, yolk and ceca of all the dead birds.

In the case of chicks in Group IIb all the birds died, four on third day and one on fourth day. Death was noticed in four birds of Group IIa and IIc. The tissues of the dead birds revealed the presence of *Salmonella* as evidenced by re-isolation.

All the birds in Group IIIa, IIIb, IIIc, IVa, IVb, and IVc died within six days PI. Re-isolation of *Salmonella* was possible from the liver, spleen, heart blood, lungs, yolk and ceca of all the dead birds.

Table 4 depicts the re-isolation of *Salmonella* from cloacal swabs collected from surviving birds on days three, seven, 11, 15, 21 and 28. Pooled cloacal swabs from Group Ia, Ic, IIa and IIc birds were positive on day three and seven. Swabs from chicks belonging to Groups Ib, IIb, IIIa, IIIc, IVa and IVc were positive on day three and then the birds died before day seven. Chicks in Group IIIb and IVb died before cloacal swab collection on day three.

At the end of experimental period the surviving chicks in Group Ia, Ic, IIa and IIc were sacrificed and the pooled organ samples were subjected for cultural

examination. *Salmonella* could be re-isolated from the cecal samples of all the groups. No *Salmonella* could be isolated from any other organ samples. The results are summarized in Table 5.

Control birds remained healthy throughout the experimental period. They were sacrificed after 28 days and visceral organs were subjected to cultural examination and there was no evidence of *Salmonella.*

Body weight gain was considerably affected in treatment groups compared to control group. In the case of Group III and IV, all chicks died before the completion of experimental period. The difference in average body weight of chicks belonging to Group I and II with the corresponding control group is given in table 6.

4.3.3 Pathogenicty in Layers

The results of pathogenicity studies by four isolates of *S. gallinarum* in layer birds are summarized in Table 7. The clinical signs and mortality were prominent and appeared early in IV route, followed by oral and IC route inoculated birds. Clinical signs observed were listlessness, inappetance, ruffled feathers, shrunken combs and sudden drop in egg production. Most of the birds which survived showed greenish-yellow diarrhea with gradual loss of weight.

In case of Group V and VI all birds survived the experimental period.

Birds belonging to Group VIIc survived the experimental period, but all the birds in Group VIIb died. One bird each died at second and fourth day and two died on the third day of inoculation. Organisms could be re-isolated from the liver, spleen, heart blood, lungs, oviduct and ceca of all dead birds while no positive isolations were obtained from the ovary of any of the dead birds.

Birds in Group VIIIa and VIIIc survived the experimental period. One bird in Group VIIIb died on fourth day of inoculation. Re-isolation was obtained from all the tissues except ovary.

Control birds remained healthy throughout the experimental period. They were sacrificed after 30 days and visceral organs were subjected to cultural examination and there was no evidence of *Salmonella.*

4.4 RE-ISOLATION OF *SALMONELLA* FROM CLINICAL SAMPLES OF EXPERIMENTALLY INFECTED BIRDS

4.4.1 Cloacal Swabs

The results of re-isolation of *Salmonella* from cloacal swabs of infected layers are depicted in Table 8.

4.4.2 Organ Samples Collected After Post Mortem

The details of re-isolation from organ samples of sacrificed layers at the end of experimental period are given in Table 9.

Pooled liver, oviduct, ovary and ceca from Group Va were positive for *Salmonella* but spleen was negative in this group. Group Vb pooled liver, spleen, oviduct and ovary were positive while pooled cecal samples gave no re-isolation. Regarding Group Vc pooled liver, spleen and oviduct were positive while ovary and ceca were negative for *Salmonella.*

In Group VIa pooled samples of liver, spleen and ovary yielded no *Salmonella* re-isolation but oviduct and ceca gave positive isolation. All the tissues were positive in Group VIb except ovary and ceca. Only liver and spleen was positive in Group VIc.

Pooled samples of liver and oviduct were positive in Group VIIa. No bird in Group VIIb survived the experimental period. Oviduct and ovary were positive in Group VIIc.

In Group VIIIa and VIIIb no re-isolation of *Salmonella* was obtained from any of the tissues but samples of liver and ceca gave positive re-isolation in Group VIIIc.

4.4.3 Eggs

4.4.3a Variation in the Number of Eggs Laid

In case of orally infected layers, reduction in egg production was noticed in the first week after inoculation. The reduction was highest in Group VIIIa. This trend in egg production continued for the second week except in Group Va where the egg production gradually increased after first week. No group reached the pre-experimental egg production during the 30 days experimental period.

Birds belonging to Groups Vb, VIb, VIIb and VIIIb showed a drastic reduction in egg production during the first week PI. Compared to all other Groups the reduction was highest in these Groups and among them most severely affected was Group VIIIb. The reducing trend in egg production continued for second week in all groups except in Group Vb which showed an increase after the first week. No group reached the pre-experimental production and in severely affected Group VIIIb by two weeks there was total cessation of egg production.

Intracloacally inoculated birds also showed reduction in egg production for the first two weeks. The reduction was most prominent in Group VIIIc. Pre-experimental production was not achieved by any of the experimental groups PI.

4.4.3b Re-isolation from Pooled Eggs

Isolation of *Salmonella* from pooled eggs collected on daily basis is given in Table 10.

Salmonella could be isolated from egg pools of day 11, 13 and 15 in Group Va, on day nine in Group Vb and on day two and 14 in Group Vc.

Similarly positive isolations were obtained from egg pools of day 10, 11 and 14 in Group VIa, on day 15, 26 and 27 in Group VIb and on day three and 21 in Group VIc.

Egg pools from Group VIIa gave positive isolation for *Salmonella* on day 11 and 21. There was no egg production in Group VIIb from second day of infection. Group VIIc eggs were positive on day five and seven.

No *Salmonella* could be isolated from Group VIIIa. There was complete cessation of egg production in Group VIIIb from second day of inoculation. *Salmonella* was isolated on day 11 from pooled egg samples of Group VIIIc.

4.5 IDENTIFICATION OF RE-ISOLATED ORGANISMS FROM EXPERIMENTAL INFECTION

The organisms isolated from clinical samples and eggs of experimentally infected birds were confirmed as *S. gallinarum* (Table 2). The organisms produced pink colonies in BGA turning the medium into pink and produced colorless colonies on MCA. All the re-isolates were Gram negative, catalase, methyl red, nitrate reduction, H_2S production and ornithine decarboxylase positive. They were negative for oxidase, indole production, Voges-Proskauer, citrate utilization, urease and phenyl alanine deaminase reactions.

4.6 GROSS PATHOLOGY

The important gross lesions observed in birds died of acute infection were generalized congestion and enlargement of all the organs. The liver was friable and on the liver and spleen there were diffused necrotic foci. Lungs were congested, oedematous and inflamed. There was severe congestion and enlargement of the vessels of the ovary. Chronically infected birds revealed pallor of breast muscles and comb with catarrhal enteritis. Day old chicks revealed severe to moderately congested unabsorbed yolk material in the abdominal cavity. There was hepatomegali, spleenomegali and pericarditis. Mice showed petechiae of the pericardium and congestion of lung. Liver and spleen showed enlargement, with congestion and focal areas of necrosis. The changes are depicted in Fig.1 to 8.

4.7 HISTOPATHOLOGY

In the myocardium there was separation, degeneration and fragmentation of muscle fibers, congestion, haemorrhage and infiltration with heterophils. Lungs showed congestion, oedema and infiltration with inflammatory cells. Liver revealed moderate to severe congestion, degeneration, multifocal necrosis and infiltrated inflammatory cells. In the spleen there was congestion and focal areas of necrosis. The changes in ovary and oviduct were very severe congestion with infiltration of inflammatory cells. Mice liver and spleen showed necrosis and microabscesses. Myocardium showed haemorrhages and separation of fibers. The changes are depicted in Fig.9 to 28.

4.8 ANALYSIS OF OUTER MEMBRANE PROTEINS (OMPs) OF *S. GALLINARUM*

4.8.1 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS - PAGE) Profiles of OMP Rich Extract

The outer membrane protein (OMP) extracts and standard protein marker were subjected to SDS-PAGE.

The medium range protein molecular weight marker yielded six bands ranging in molecular weights from 97.4 kDa to 14.3 kDa. The OMP profiles of all the three *S. gallinarum* local isolates and the reference strain BGL were almost identical. There were about 13 protein bands with approximate molecular weight ranging from 13 to 110 kDa as determined by Biorad software compared with molecular weights of the medium range protein molecular weight marker. Molecular weights of the 13 different polypeptide bands were 109.24, 96.43, 83.73, 71.68, 64.54, 56.74, 51.41, 48.46, 38.78, 37.15, 34.23, 32.53 and 18.96 kDa. Five protein bands with approximate molecular weight of 109.24, 71.68, 64.54, 48.46 and 38.78 kDa were thicker than others, suggesting that they were the major OMPs (Fig. 29).

4.9 ANALYSIS OF HOMOGENEITY/HETEROGENEITY OF BACTERIAL ISOLATES BY SEROLOGY

4.9.1 AGID

On viewing the gel against diffuse light, lines of identity were observed with all four isolates using both BGL and QS 1 hyper immune serum (Fig. 30 and 31).

Table 1. Antibiogram of *Salmonella* isolates

Antibiotic/Antibacterial agents	Isolates			
	BGL	PS-8	QS-1	X-4
Amoxy clav	S	S	S	S
Cephalexin	S	S	S	S
Chloramphenicol	S	S	S	S
Ciprofloxacin	S	S	S	S
Clindamycin	R	R	R	R
Cloxacillin	R	R	R	R
Co-trimoxazole	R	R	R	S
Enrofloxacin	S	S	S	S
Erythromycin	R	R	R	R
Gentamicin	S	S	S	S
Nalidixic acid	R	R	R	R
Penicillin-G	R	R	R	R
Tetracycline	S	S	S	S

R - Resistant
S – Susceptible

Table 2. Tests for identification of re-isolated *Salmonella*

Tests	Isolates			
	BGL	PS 8	QS 1	X 4
Gram's reaction	G-ve	G-ve	G-ve	G-ve
Catalase	+	+	+	+
Oxidase	-	-	-	-
Indole	-	-	-	-
Methyl Red	+	+	+	+
Voges-Proskauer	-	-	-	-
Citrate Utilization	-	-	-	-
Nitrate Reduction	+	+	+	+
Urease	-	-	-	-
Phenyl Alanine Deaminase	-	-	-	-
H_2S production	+	+	+	+
Ornithine decarboxylase	+	+	+	+

Table 3. Pathogenicity study in day-old chicks

Bacterial isolate used	Route of inoculation	No: of chicks/ route	Mortality after inoculation (hours)							Re-isolation					
			24	48	72	96	120	144	Total	Liver	Spleen	Heart blood	Lungs	Yolk	Ceca
Group I	Group Ia	5	-	-	-	-	-	-	-	*NT	*NT	*NT	*NT	*NT	*NT
	Group Ib	5	1	1	2	-	1	-	5	+	+	+	+	+	+
	Group Ic	5	-	-	-	-	-	-	-	*NT	*NT	*NT	*NT	*NT	*NT
Group II	Group IIa	5	-	-	-	2	2	-	4	+	+	+	+	+	+
	Group IIb	5	-	-	4	1	-	-	5	+	+	+	+	+	+
	Group IIc	5	-	-	-	2	2	-	4	+	+	+	+	+	+
Group III	Group IIIa	5	-	1	1	-	2	1	5	+	+	+	+	+	+
	Group IIIb	5	-	5	-	-	-	-	5	+	+	+	+	+	+
	Group IIIc	5	-	-	-	2	2	1	5	+	+	+	+	+	+
Group IV	Group IVa	5	-	-	1	2	2	-	5	+	+	+	+	+	+
	Group IVb	5	2	3	-	-	-	-	5	+	+	+	+	+	+
	Group IVc	5	-	-	1	1	3	-	5	+	+	+	+	+	+

*NT – Not Tested

Table 4. Re-isolation of *Salmonella* from pooled cloacal swabs of survived chicks

Bacterial isolate used	Route of inoculation	Day 3		Day 7		Day 11		Day 15		Day 21		Day 28	
		No. of chicks from which pooled swabs collected	Positive/ negative	No. of chicks from which pooled swabs collected	Positive/ negative	No. of chicks from which pooled swabs collected	Positive/ negative	No. of chicks from which pooled swabs collected	Positive/ negative	No. of chicks from which pooled swabs collected	Positive/ negative	No. of chicks from which pooled swabs collected	Positive/ negative
Group I	Group Ia	5	+	5	+	5	-	5	-	5	-	5	-
	Group Ib	1	+	-	-	-	-	-	-	-	-	-	-
	Group Ic	5	+	5	+	5	-	5	-	5	-	5	-
Group II	Group IIa	5	+	1	+	1	-	1	-	1	-	1	-
	Group IIb	1	+	-	-	-	-	-	-	-	-	-	-
	Group IIc	5	+	1	+	1	-	1	-	1	-	1	-
* Group III	Group IIIa	3	+	-	-	-	-	-	-	-	-	-	-
	Group IIIb	-	-	-	-	-	-	-	-	-	-	-	-
	Group IIIc	5	+	-	-	-	-	-	-	-	-	-	-
* Group IV	Group IVa	4	+	-	-	-	-	-	-	-	-	-	-
	Group IVb	-	-	-	-	-	-	-	-	-	-	-	-
	Group IVc	4	+	-	-	-	-	-	-	-	-	-	-

* All birds in these groups died within six days PI

Table 5. Re-isolation of *Salmonella* from organ pool of sacrificed chicks at the end of experimental period

Isolate	Route	No: of chicks examined	Liver	Spleen	Heart blood	Ceca
Group I	Group Ia	5	-	-	-	+
	Group Ic	5	-	-	-	+
Group II	Group IIa	1	-	-	-	+
	Group IIc	1	-	-	-	+

All chicks belonging to Group III, Group IV, Group Ib and Group IIb died before the end of experimental period.

Table 6. Difference in body weight of survived chicks at the end of experimental period

Isolate	Route	Treatment group Average weight (Grams)	Control group Average weight (Grams)
Group I	Group Ia	140	175.5
	Group Ic	150	183.33
Group II	Group IIa	50	166.67
	Group IIc	100	162.5

All chicks belonging to Group III, Group IV, Group Ib and Group IIb died before the end of experimental period.

Table 7. Pathogenicity study in layers

Bacterial isolate used	Route of inoculation	No: of birds/ route	Mortality after inoculation (hours)							Total No. of isolation/Total No. examined						
			24	48	72	96	120	148	Total	Liver	Spleen	Heart	Lungs	Oviduct	Ovary	Ceca
Group V	Group Va	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Group Vb	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Group Vc	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group VI	Group VIa	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Group VIb	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Group VIc	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group VII	Group VIIa	4	-	-	-	1	-	-	1	1/1	1/1	1/1	1/1	1/1	0/1	1/1
	Group VIIb	4	-	1	2	1	-	-	4	4/4	4/4	4/4	4/4	4/4	0/4	4/4
	Group VIIc	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group VIII	Group VIIIa	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Group VIIIb	4	-	-	-	1	-	-	1	1/1	1/1	1/1	1/1	1/1	0/1	1/1
	Group VIIIc	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 8. Re-isolation of *Salmonella* from cloacal swabs of layers

Group V												
Route	Group Va				Group Vb				Group Vc			
Bird No.	3978	3858	4444	4066	3894	3826	3892	3760	3744	4396	3736	4276
Day3	+	+	+	+	+	-	-	+	-	-	+	+
Day7	-	+	-	-	-	-	-	+	+	-	-	-
Day 11	-	-	-	-	-	-	+	+	-	-	+	-
Day 15	+	-	+	+	-	-	-	+	-	-	-	+
Day 21	-	+	-	-	-	-	-	-	-	-	-	-
Day 28	-	+	+	-		-	+	-	+	-	-	+
Group VI												
Route	Group VIa				Group VIb				Group VIc			
Bird No.	4703	3789	4291	4021	4068	4283	4246	4234	4107	3784	3865	4244
Day 3	+	+	+	-	+	+	+	-	-	+	+	+
Day 7	-	-	-	-	-	+	+	-	-	-	-	-
Day 11	-	+	-	-	-	-	-	-	-	+	+	-
Day 15	+	-	-	-	-	-	-	+	-	-	-	-
Day 21	-	-	+	-	-	-	-	-	-	-	-	-
Day 28	-	-	-	-	-	-	+	+	-	-	-	+
Group VII												
Route	Group VIIa				Group VIIb				Group VIIc			
Bird No.	4227	3876	4317	4359	3731	4073	4352	4080	4383	3857	3756	4372
Day 3	+	+	+	-	-	*NT	*NT	*NT	-	+	+	-
Day 7	-	-	*NT	-	*NT	*NT	*NT	*NT	-	-	-	-
Day 11	-	-	*NT	-	*NT	*NT	*NT	*NT	+	-	-	-
Day 15	+	-	*NT	-	*NT	*NT	*NT	*NT	-	-	+	+
Day 21	-	-	*NT	+	*NT	*NT	*NT	*NT	-	-	-	-
Day 28	-	-	*NT	-	*NT	*NT	*NT	*NT	-	-	+	-
Group VIII												
Route	Group VIIIa				Group VIIIb				Group VIIIc			
Bird No.	3750	4126	4235	3852	3829	4315	4434	3766	4274	4254	3785	4352
Day 3	+	-	-	-	+	+	-	-	+	+	+	+
Day 7	+	+	-	+	*NT	-	-	-	-	-	+	-
Day 11	+	+	+	+	*NT	-	-	-	+	-	+	-
Day 15	-	-	-	-	*NT	-	-	-	-	+	-	-
Day 21	+	-	-	-	*NT	-	-	-	-	-	-	-
Day 28	-	-	-	-	*NT	-	-	+	-	-	-	-

*NT – Not Tested

Table 9. Re-isolation of *Salmonella* from pooled organ samples of sacrificed layers at the end of experimental period

Isolate	Route	No: of birds in the pool	Liver	Spleen	Oviduct	Ovary	Ceca
Group V	Group Va	4	+	-	+	+	+
	Group Vb	4	+	+	+	+	-
	Group Vc	4	+	+	+	-	-
Group VI	Group VIa	4	-	-	+	-	+
	Group VIb	4	+	+	+	-	-
	Group VIc	4	+	+	-	-	-
Group VII	Group VIIa	3	+	-	+	-	-
	Group VIIb	-	-	-	-	-	-
	Group VIIc	4	-	-	+	+	-
Group VIII	Group VIIIa	4	-	-	-	-	-
	Group VIIIb	3	-	-	-	-	-
	Group VIIIc	4	+	-	-	-	+

Table 10. Re-isolation of *Salmonella* from pooled eggs

	Group V			Group VI			Group VII			Group VIII		
	+ or -			+ or -			+ or -			+ or -		
	Va	Vb	Vc	VIa	VIb	VIc	VIIa	VIIb	VIIc	VIIIa	VIIIb	VIIIc
Day1	-	-	-	-	-	-	-	-	-	-	-	-
Day 2	-	*NT	+	-	*NT	-	*NT	*NT	-	-	*NT	-
Day 3	-	*NT	-	-	*NT	+	-	*NT	-	-	*NT	-
Day 4	-	-	-	-	*NT	-	-	*NT	*NT	*NT	*NT	-
Day 5	*NT	-	-	-	*NT	-	-	*NT	+	*NT	*NT	-
Day 6	-	-	-	-	*NT	-	-	*NT	-	-	*NT	-
Day 7	-	-	-	-	*NT	*NT	*NT	*NT	+	*NT	*NT	-
Day 8	-	-		-	*NT	-	-	*NT		*NT	*NT	-
Day 9	-	+	-	-	*NT	-	-	*NT	-	*NT	*NT	-
Day 10	-	-	-	+	*NT	-	-	*NT	-	*NT	*NT	*NT
Day 11	+	-	-	+	*NT	-	+	*NT	-	*NT	*NT	+
Day 12	-	-	-	-	*NT	*NT	-	*NT	-	*NT	*NT	*NT
Day 13	+	-	-	-	*NT	-	-	*NT	-	*NT	*NT	*NT
Day 14	-	-	+	+	*NT	-	-	*NT	-	*NT	*NT	*NT
Day 15	+	-		-	+	-	-	*NT	-	*NT	*NT	-
Day 16	-	-	-	-	*NT	-	-	*NT	-	*NT	*NT	-
Day 17	-	-	-	-	*NT	*NT	-	*NT	-	*NT	*NT	-
Day 18	-	-		-	*NT	-	-	*NT	-	*NT	*NT	-
Day 19	*NT	-	-		-	*NT	-	*NT	-	-	*NT	-
Day 20	-	-	-	-	-	-	-	*NT	-	-	*NT	-
Day 21	-	-	-		-	+	+	*NT	-	-	*NT	-
Day 22	-	-	-	-	-	-	-	*NT	-	-	*NT	*NT
Day 23	*NT	-	-	-	-		-	*NT	-	-	*NT	-
Day 24	-	-	-	-	-	-	-	*NT	-	-	*NT	-
Day 25	-	-	-	-	-	-	-	*NT	-	-	*NT	-
Day 26	-	*NT	-	-	+	-	-	*NT	-	-	*NT	-
Day 27	-	*NT	-	-	+	-	-	*NT	-	*NT	*NT	-
Day 28	*NT	-	-	-	-	-	-	*NT	*NT	-	*NT	-
Day 29	*NT	-	-		-	-	-	*NT	-	-	*NT	-
Day 30	*NT	-	-	-	-	-	-	*NT	-	-	*NT	-

*NT – Not Tested

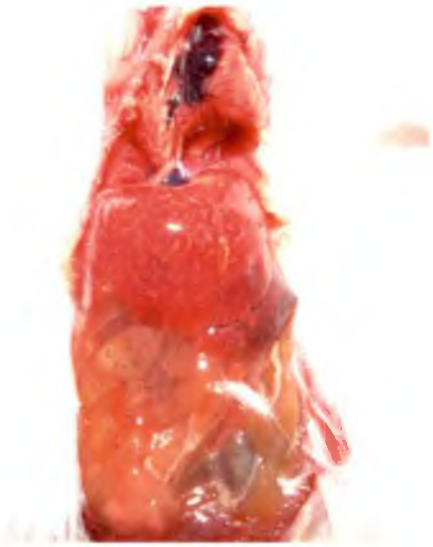


Fig. 1

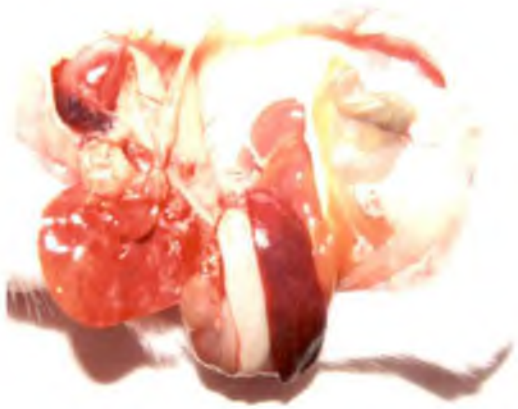


Fig. 2

Fig. 3

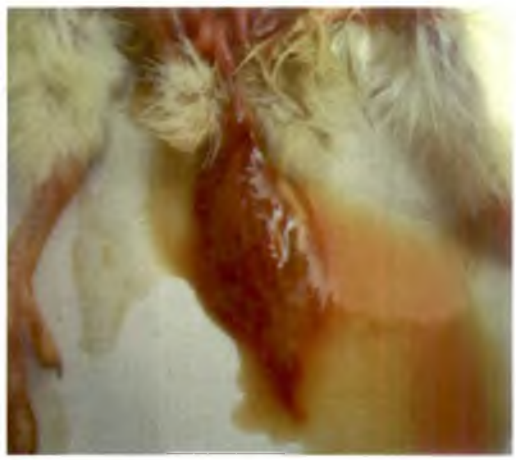


Fig. 4

Figure 1: Mice infected with *Salmonella* - Liver showing diffused necrotic foci

Figure 2: Mice infected with *Salmonella* - Spleen showing splenomegaly

Figure 3: Chick - Liver - Degeneration and necrosis

Figure 4: Chick - Yolk sac - Omphalitis

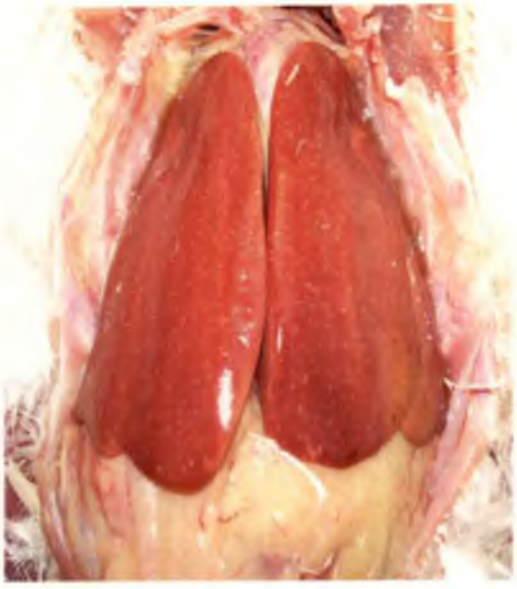
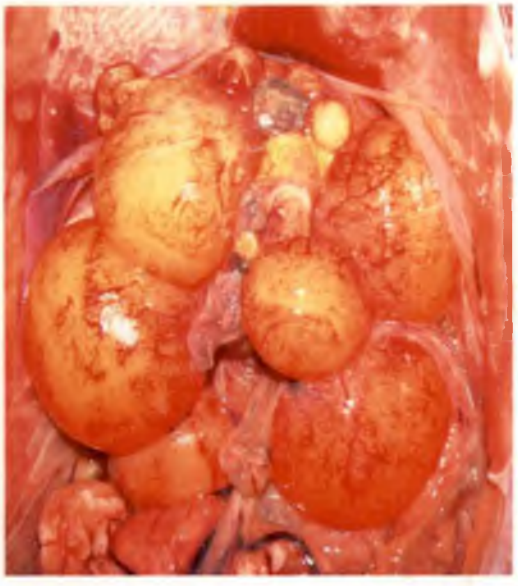


Fig. 5

Fig. 6



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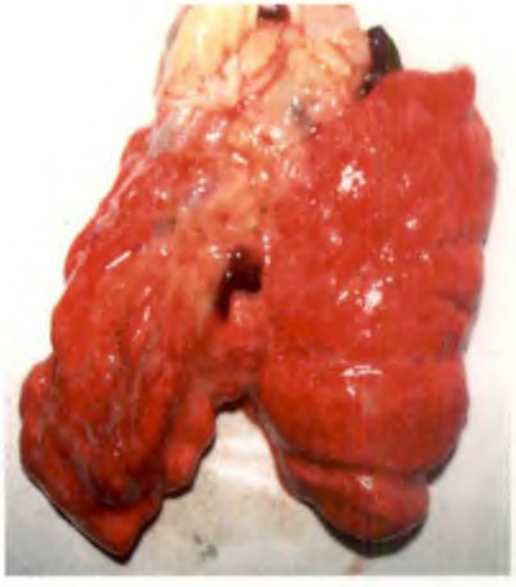


Fig. 8

Figure 5: Layer - Liver - Scattered necrotic foci

Figure 6: Layer - Spleen - Splenomegaly with diffused necrotic spots

Figure 7: Layer - Ovary - Engorged blood vessels

Figure 8: Layer - Lungs - Pulmonary congestion and oedema

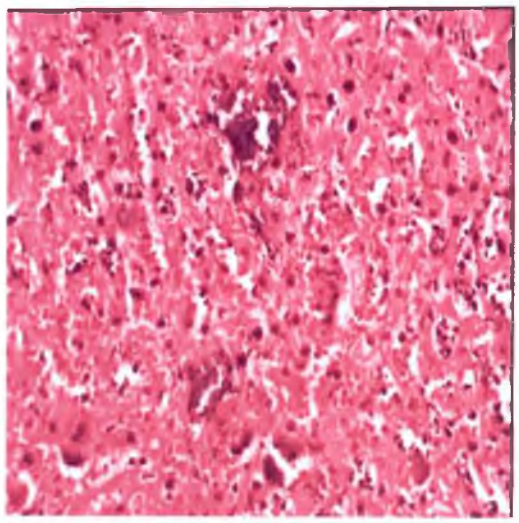


Fig. 9

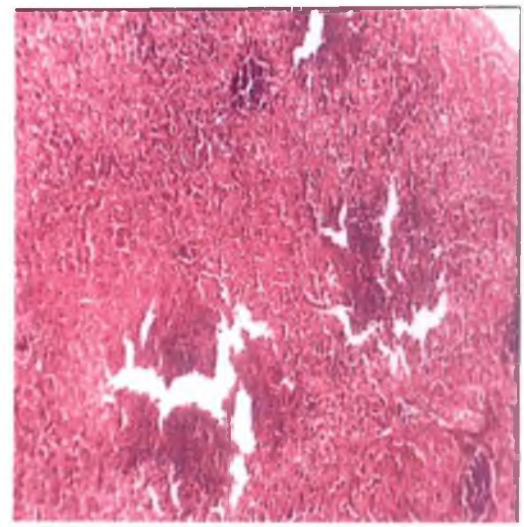


Fig. 10

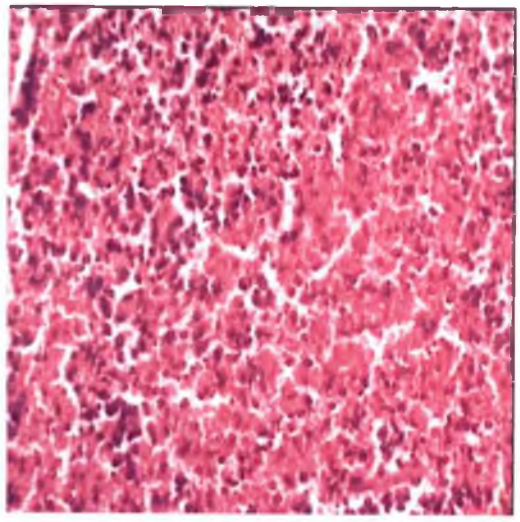


Fig. 11

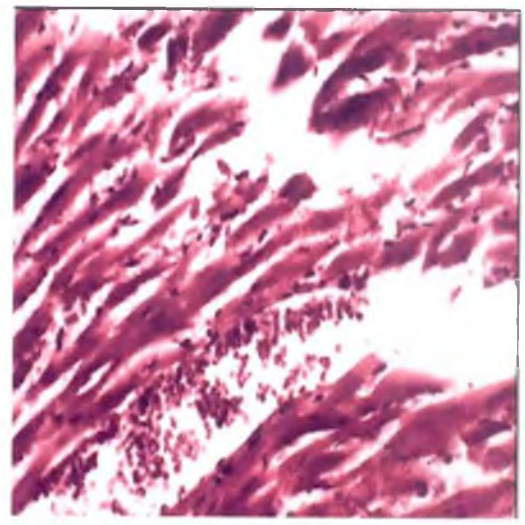


Fig. 12

Figure 9: Mice - Liver - Microabscesses and haemorrhage - H & E X 400

Figure 10: Mice - Spleen - Abscesses - H & E x 100

Figure 11: Mice - Spleen - Haemorrhage and depletion of lymphocytes - H & E x 400

Figure 12: Mice - Myocardium - Haemorrhage and separation of fibers - H & E x 400

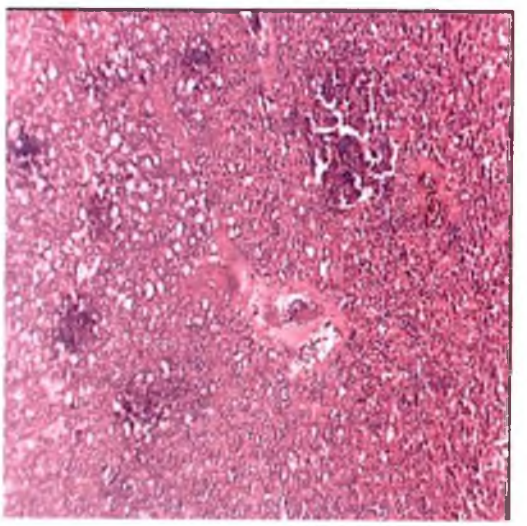


Fig. 13

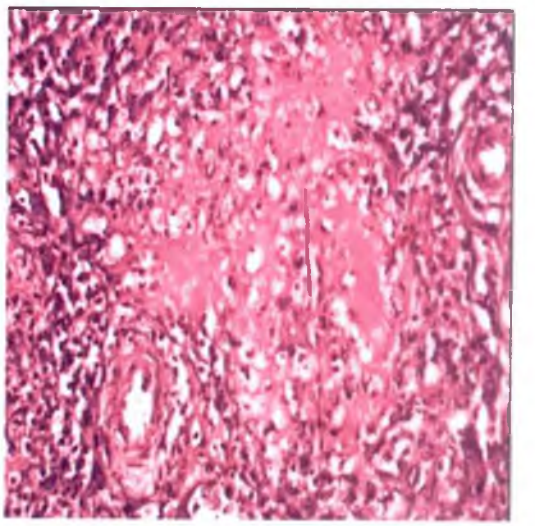


Fig. 14

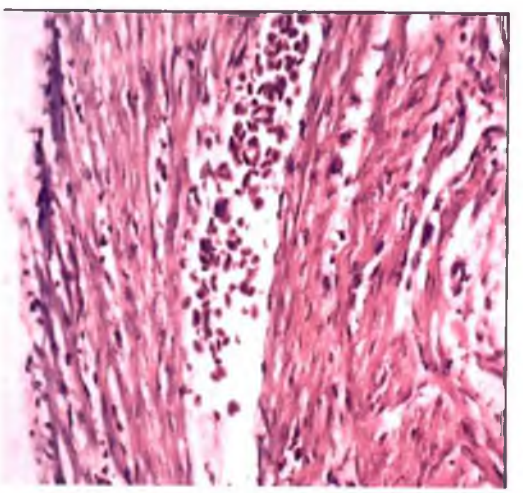


Fig. 15

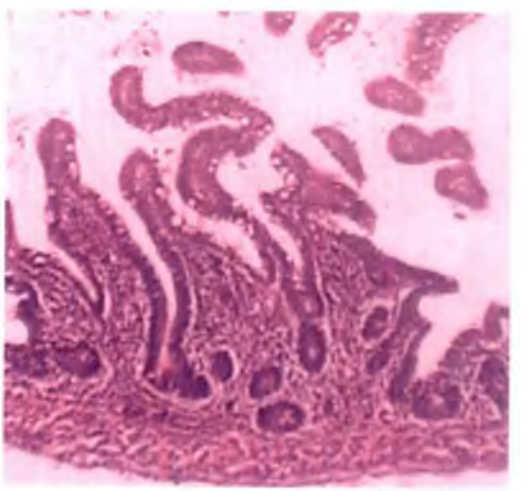


Fig. 16

Figure 13: Chick - Liver - Microabscesses and vacuolar changes - H & E x 100

Figure 14: Chick - Spleen - Necrosis and depletion of lymphoid cells - H & E x 400

Figure 15: Chick - Myocardium - Haemorrhage and infiltration of inflammatory cells - H & E x 400

Figure 16: Chick - Ceca - Goblet cell hyperplasia and infiltration of inflammatory cells in submucosa - H & E x 100

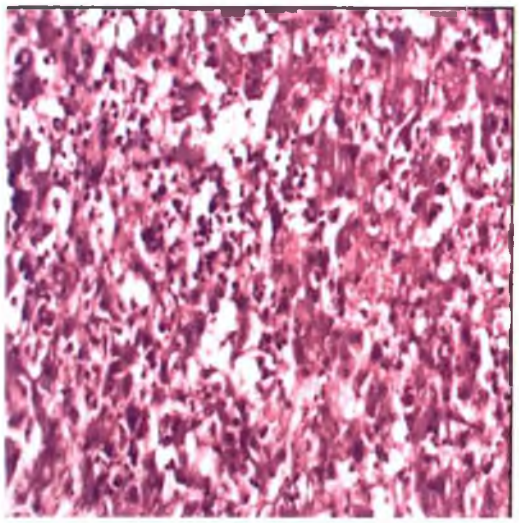
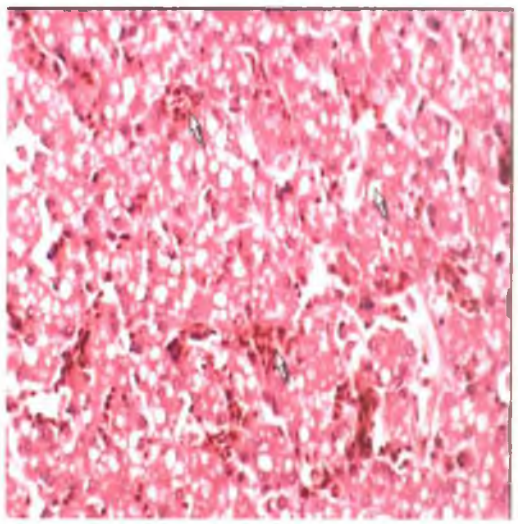


Fig. 17



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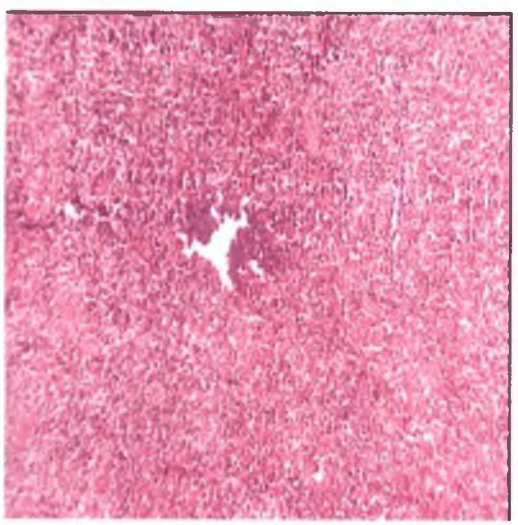

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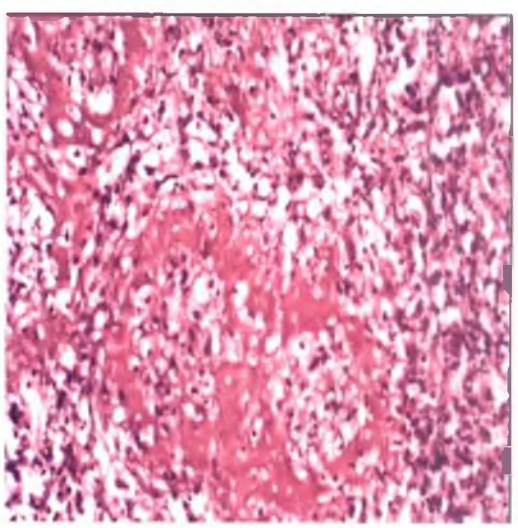


Fig. 20

Figure 17: Layer - Liver - Inflammation and vacoular changes - H & E x 400

Figure 18: Layer - Liver - Haemorrhage in parenchyma with vacoular degeneration - H & E x 400

Figure 19: Layer - Spleen - Necrotic foci - H & E x 100

Figure 20: Layer - Spleen - Depletion of lymphocytes - H & E x 400

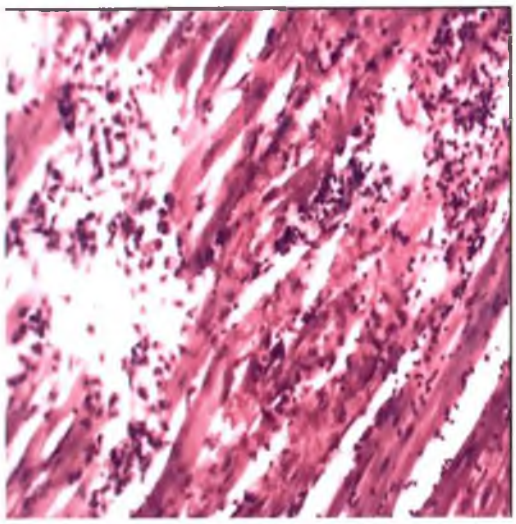
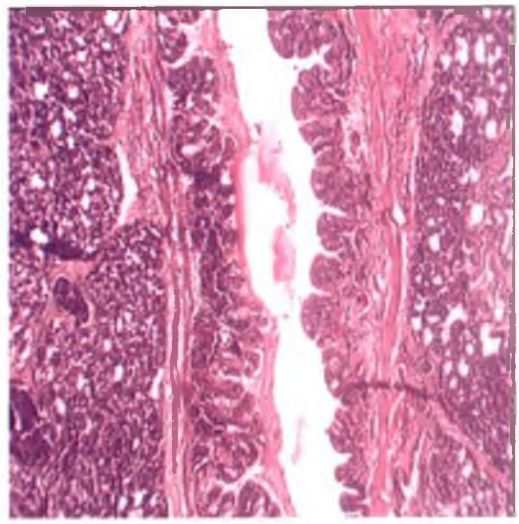


Fig. 21



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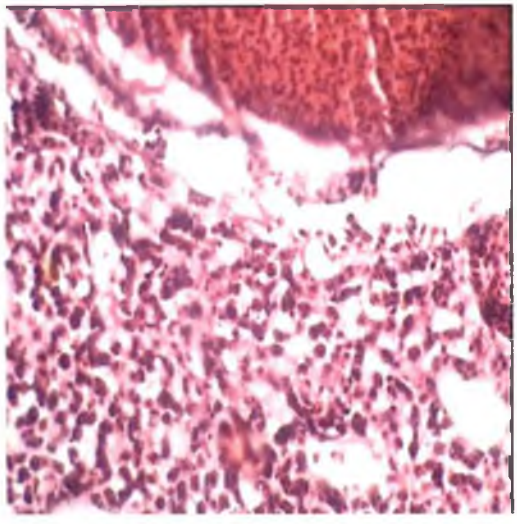

Fig. 23

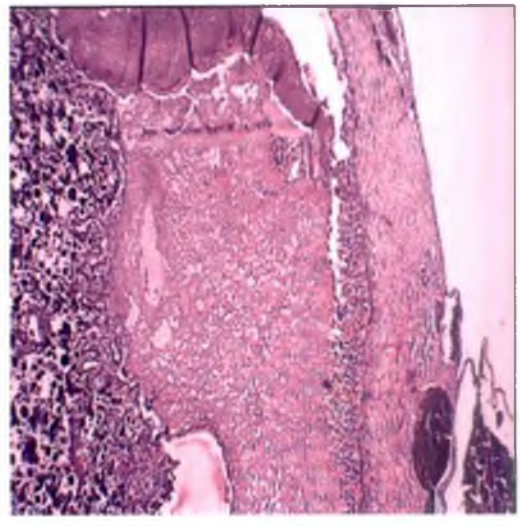

Fig. 24

Figure 21: Layer - Myocardium - Fragmentation of muscle fibers, inflammatrory cells and haemorrhage - H & E x 400

Figure 22: Layer - Lungs - Hyperplasia of bronchial epithelium with exudate - H & E x 400

Figure 23: Layer - Lungs - Inflammatory cells and engorged vessels - H & E x 400

Figure 24: Layer - Ovary - Follicular membrane showing inflammatory cells - H & E x 40

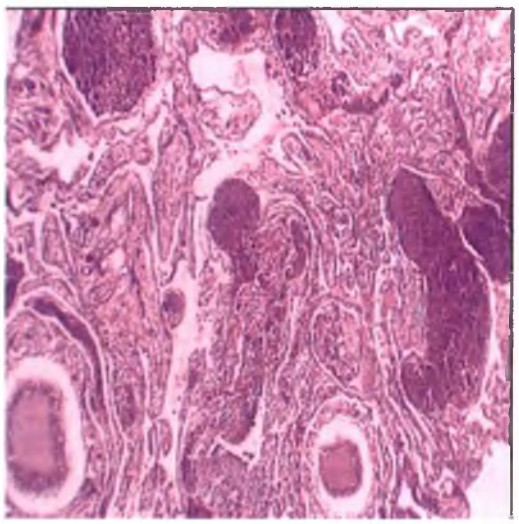

Fig. 25

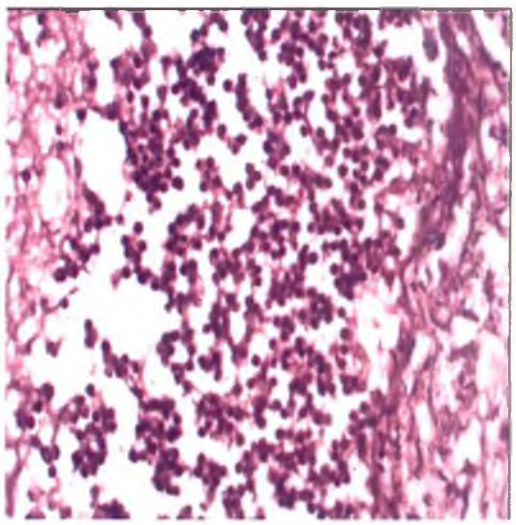

Fig. 26

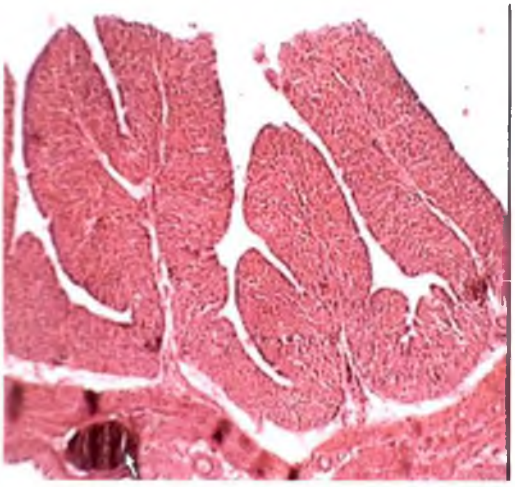

Fig. 27

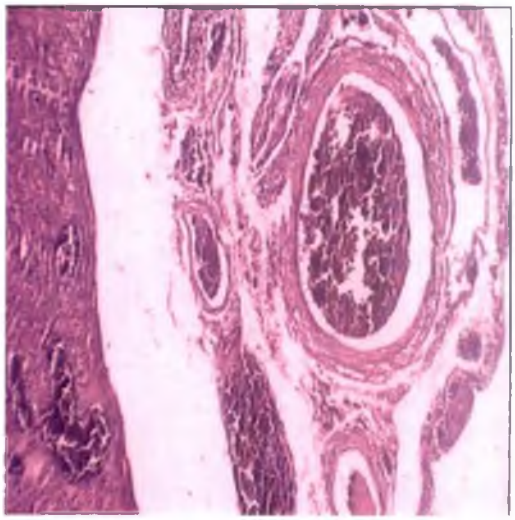

Fig. 28

Figure 25: Layer - Ovary - Engorged vessels - H & E x 100

Figure 26: Layer - Ovary - Presence of inflammatory cells - H & E x 400

Figure 27: Layer - Oviduct - Hyperplasia of ductal epithelium with congested vessels - H & E x 400

Figure 28: Layer - Oviduct - Engorged blood vessels and inflammatory cells - H & E x 400

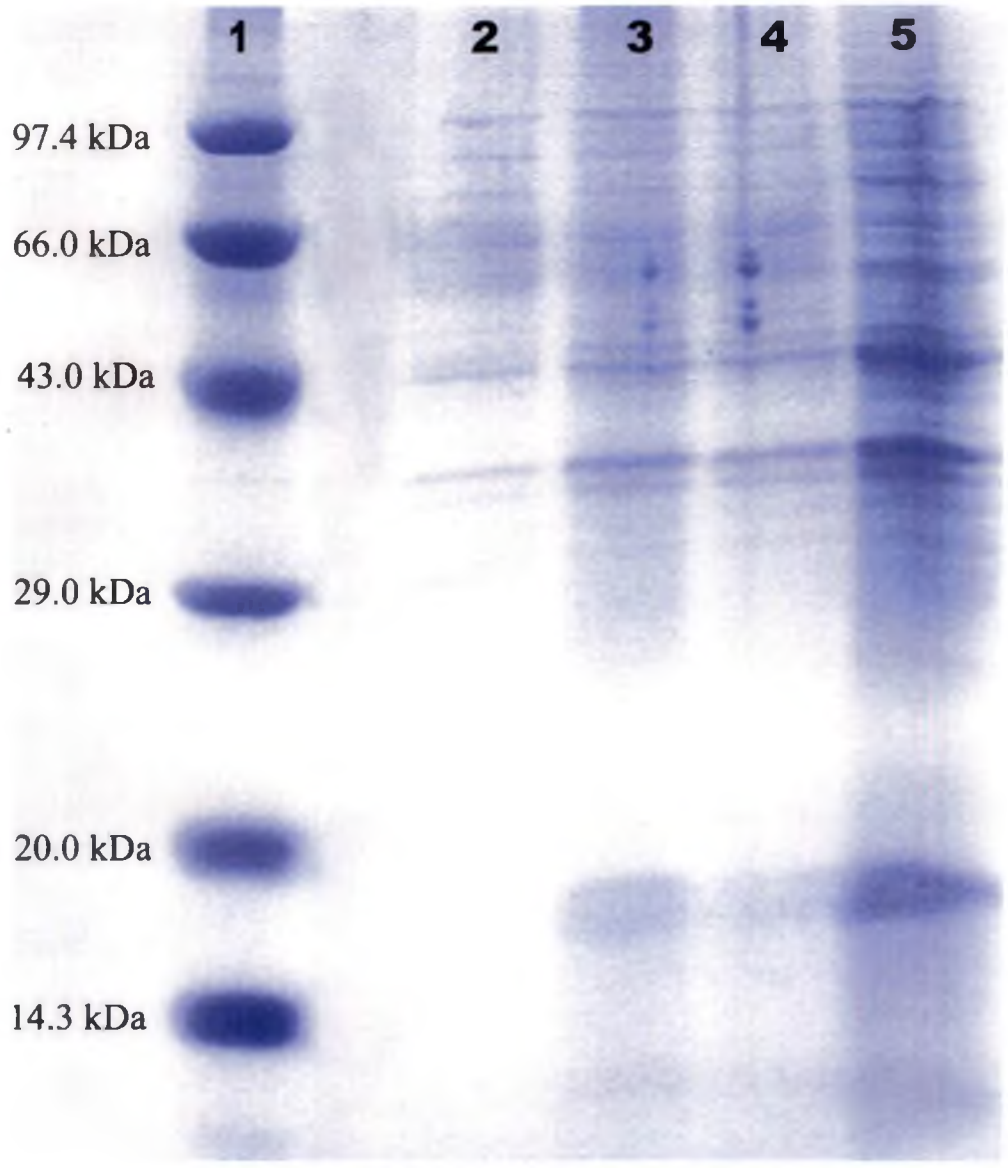

Fig. 29 SDS - PAGE analysis of *S.gallinarum* Outer Membrane Proteins

Lane 1: Medium range protein molecular weight marker

Lane 2: X 4

Lane 3: QS 1

Lane 4: PS 8

Lane 5: BGL

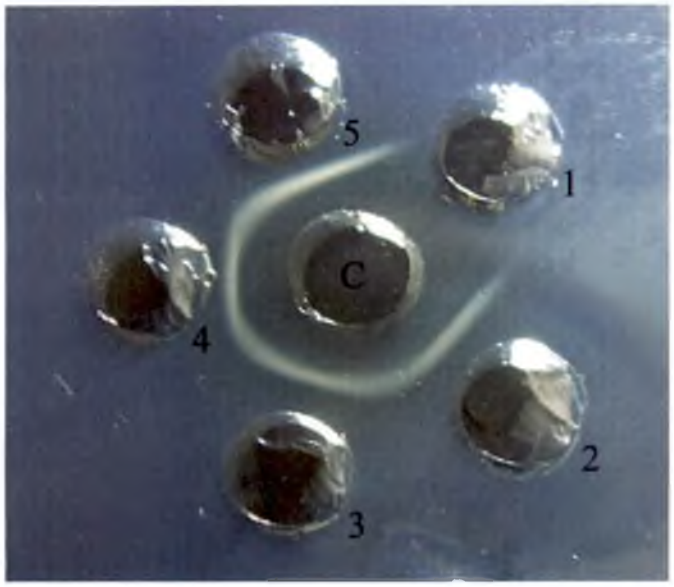

Fig. 30 AGID using hyperimmune serum against *S.gallinarum* BGL isolate

C - Hyperimmune serum against BGL isolate
1 - Negative control
2 - Whole cell protein BGL
3 - Whole cell protein PS 8
4 - Whole cell protein QS 1
5 - Whole cell protein X 4

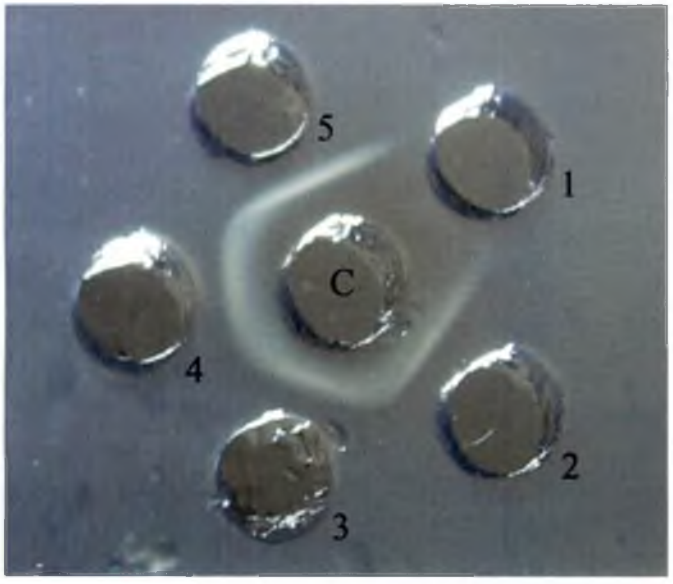

Fig. 31 AGID using hyperimmune serum against *S.gallinarum* QS 1 isolate

C - Hyperimmune serum against QS 1 isolate
1 - Negative control
2 - Whole cell protein BGL
3 - Whole cell protein PS 8
4 - Whole cell protein QS 1
5 - Whole cell protein X 4

Discussion

5. DISCUSSION

Fowl typhoid, caused by *S. gallinarum*, is one of the major diseases of economic importance for poultry, distributed world wide, including Indian sub-continent. *Salmonella* infection in domestic poultry has been reported from almost all parts of India. Among them, *S. gallinarum* was the most frequently isolated serotype, associated with avian salmonellosis. Determining the antigenic homogeneity/heterogeneity and pathogenicity of different isolates is an important step in devising proper control measures against FT.

5.1 ANTIBIOGRAM

All the four *Salmonella* isolates were subjected to antibiotic sensitivity testing. All the isolates were sensitive to amoxy clav, cephalexin, chloramphenecol, ciprofloxacin, enrofloxacin, gentamicin and tetracycline. All of them were resistant to penicillin-G, nalidixic acid, erythromycin, cloxacillin and clindamycin. These patterns were in accordance with Sujatha *et al.* (2003) and Shukla and Singh (2001).

Three isolates *viz.*, BGL, PS 8 and QS 1 were resistant to co-trimoxazole, but isolate X 4 was susceptible. Poppe *et al.* (1995) had reported that out of 2690 *Salmonella* isolates belonging to 52 serovars, only 1.7 per cent showed resistance to trimethoprim-sulphomethoxazole combination. The susceptibility of X 4 isolate to co-trimoxazole might be due to the absence of sulpha resistance plasmid. But this could not be ascertained as plasmid isolation and its role in antibiotic resistance was not studied.

The antibiotic sensitivity pattern revealed by the four isolates were identical, except for co-trimoxazole.

5.2 PATHOGENICITY IN MICE

Except for the reference strain BGL, the other three isolates were found to be pathogenic to mice. The most pathogenic was the isolate PS 8 which killed mice within 24 h, followed by QS 1 and X 4 which killed mice in 72 and 96 h respectively. *Salmonella gallinarum* isolate BGL was unable to kill or produce any clinical signs in mice even after seven days post infection. Kokosharov and Phetisova (2002) reported that eight out of 12 *S. gallinarum* strains isolated from dead hens of different poultry farms were lethal to adult white mice after intra-peritoneal inoculation.

5.3 PATHOGENICITY IN DAY OLD CHICKS

The clinical signs and mortality were predominant and appeared early in IM route, followed by oral and IC route inoculated chicks. Singh *et al.* (1996) reported that mortality due to natural and experimental infection with *S. gallinarum* in chickens varied from zero to 100 per cent, which could be attributed to wide variation in virulence of different *S gallinarum* strains. The major clinical signs observed were somnolence, poor growth, weakness, inappetence and whitish diarrhoea.

Isolates QS 1 and X 4 were found to be most pathogenic to day-old chicks, causing 100 per cent mortality through all the three routes of inoculation. Even though isolates BGL and PS 8 were pathogenic to day-old chicks by IM route, isolate BGL was least pathogenic, causing no mortality in oral and IC inoculated groups.

Salmonella could be isolated from cloacal swabs collected on days three and seven in surviving chicks. In birds which survived the experimental period re-isolation of salmonellae was possible from ceca even though no other organs yielded isolation. Dhillon *et al.* (2001) also reported that cecal pouches were the ideal organs for re-isolation of salmonellae, compared with other organs.

Survived birds showed considerable reduction in body weight after 28 days of experimental period. Dhillon *et al.* (2001) reported 1.8 to 12.6 per cent reduction in mean body weight in inoculated groups, compared to control groups.

5.4 PATHOGENICITY IN LAYERS

The clinical signs and mortality were prominent and appeared early in IV route, followed by oral and IC route inoculated birds. Clinical signs observed were listlessness, inappetence, ruffled feathers, shrunken combs and sudden drop in egg production, with most affected birds exhibiting greenish-yellow diarrhea with gradual weight loss. These findings were similar to those of Nakamura *et al.* (1992), Joshi *et al.* (2000) and Rahman *et al.* (2002).

The cloacal swabs from infected surviving birds were examined on day three, seven, eleven, fifteen, twenty one and twenty eight. Birds belonging to all the groups, except group VIIb where all birds died within three days PI, showed fecal shedding of *Salmonella* which was intermittent. This was in accordance with Shivaprasad *et al.* (1990).

In orally challenged birds, the highest fecal shedding was seen with birds inoculated with BGL, followed by X 4, PS 8 and QS 1. One bird belonging to PS 8 oral group did not show fecal shedding at all during the experimental period.

In IV inoculated birds such comparisons could not be made since there was early mortality in Group VIIb and Group VIIIb.

In the IC inoculated birds highest fecal shedding was seen with Group VIIIc, followed by Group Vc and then Group VIc and Group VIIc, with equal number of shedders.

From the above results it could be seen that isolate QS 1 was the most virulent strain of *S. gallinarum* which caused 100 per cent mortality in all IV inoculated birds, eventhough shedding of the organism was not at the highest rate. Fierer and Guiney (2001) reported that the important virulence determinants

of *Salmonella* were differential expression of various fimbriae, SPI 1, SPI 2, SPI 3 and genes like *shd* A associated with fecal shedding that were individual genes that encoded different *Salmonella* lineages of individual strains. Gupta *et al.* (1996) opined that the invasiveness and lethality of *S. gallinarum* isolates were two different characteristics. Lethal organisms were highly invasive also but all highly invasive organisms were not lethal. High invasiveness could lead to persistence of organisms for longer time.

Shivaprasad *et al.* (1990) reported that mean fecal shedding was highest for IV inoculated groups. In this experiment it was difficult to arrive at a conclusion since there was heavy mortality in Group VIIb and Group VIIIb. In case of isolate BGL, highest fecal shedding was seen with orally inoculated group followed by IV group but with PS 8 highest fecal shedding was observed in IV route inoculated birds.

The layer birds were sacrificed at the end of the experimental period and re-isolation of *Salmonella* was attempted from the organ pool. It was found that the highest isolation was obtained from liver and oviduct, then spleen, ovary and ceca with equal number of isolations. This was against the findings of Gast and Beard (1990) who experimentally infected 20 to 22 week-old hens with *Salmonella* orally and horizontally and re-isolated it from 58 per cent ceca, 51 per cent of liver, 47 per cent of spleens and 17 per cent of oviduct sampled during first five weeks after exposure. Among the four isolates studied the highest number of re-isolation in all the three routes was obtained for *S. gallinarum* isolate BGL.

Comparing the egg production in challenged birds, drastic reduction was seen in birds inoculated through IV route. This was in accordance to Sivaprasad *et al.* (1990) who reported highest drop in mean egg production among IV route inoculated birds. Regarding the other two routes *viz.*, oral and cloacal, highest reduction was seen for the isolate X 4.

Salmonella could be re-isolated from pooled eggs of groups Va, Vb, Vc, VIa, VIb, VIc, VIIa, VIIc and VIIIc. No re-isolations could be made from groups VIIb, VIIIa and VIIIb. This might be because the number of eggs obtained from these three groups during the experimental period was less, compared to other groups which gave positive isolation. No significant difference could be noticed between different routes of inoculation and re-isolation from egg. Shivprasad *et al.* (1990) had reported that the highest egg shell contamination was obtained with intra cloacal route, while highest internal egg contamination was obtained with IV route of inoculation. In this study such comparisons could not be made since no separate examination of shell, white and yolk was conducted. The number of positive isolation from eggs were higher which might be due to the fecal contamination of egg shells in this experiment.

5.5 IDENTIFICATION OF REISOLATED ORGANISM FROM EXPERIMENTAL INFECTION

The re-isolates were confirmed as *S. gallinarum*. All the organisms were Gram negative, catalase, methyl red, nitrate reduction, H_2S production and ornithine decarboxylase positive and were negative for oxidase, indole production, Voges-Proskauer, citrate utilization, urease and phenyl alanine deaminase reactions. These findings were in accordance with Shivhare *et al.* (2000) and Mutharamalingam (2005).

5.6 GROSS PATHOLOGY

Gross pathological changes observed in acute infection were enlargement of liver and spleen with diffused white necrotic spots, congestion of heart and lungs. Day-old chicks revealed inflamed, unabsorbed yolk material in the abdominal cavity. Similar type of lesions were observed with all the four isolates which caused death on experimental infection. These findings in *Salmonella* experimental infection were in accordance with those of other workers (Verma, 1968; Rahman *et al.*, 1997; Shah *et al.*, 2000).

5.7 HISTOPATHOLOGY

Histopathological changes observed in acute infection were congestion, necrosis and vacuolar degeneration in liver, infiltration of inflammatory cells and RBC's in between myocardial fibers, pulmonary oedema and congestion, congestion and infiltration of inflammatory cells in ovary and sub mucosa of intestine. Similar findings had been reported by Joshi *et al.* (2000) Prakash *et al.* (2002) and Prasanna *et al.* (2001).

5.8 SDS-PAGE FOR DETECTION OF ANTIGENICITY OF DIFFERENT *SALMONELLA* ISOLATES

The OMP extracts of all the three local isolates and reference strain BGL were subjected to SDS-PAGE analysis. The SDS-PAGE profile of the OMP extracts of local isolates obtained were identical to that exhibited by reference strain BGL. Thirteen different polypeptide bands of molecular weight ranging between 110 to 18 kDa were clearly identified for all the four isolates and their molecular weight was calculated using Biorad software compared to standard medium range protein molecular weight marker. Five protein bands were thicker than others suggesting that they were the major OMP's which were also identical in all the isolates. It could be concluded that all the three local isolates and the reference strain BGL exhibited high degree of antigenic homogeneity.

The findings were similar to those of Sujatha *et al.* (2003) who obtained 19 to 26 bands having molecular weight ranging from 14 kDa to 97 kDa on SDS PAGE analysis of *S. gallinarum* field isolate antigen. Lee *et al.* (2005) had also reported OMP profile of *S. gallinarum* on SDS-PAGE as 15 polypeptide bands of molecular weight ranging from 32 kDa to 99 kDa.

5.9 ANALYSIS OF HOMOGENITY/HETEROGENITY OF BACTERIAL ISOLATES BY SEROLOGY

Agar gel immunodiffusion was carried out to find out the antigenic homogeneity/heterogeneity of the four local isolates and the reference isolate. Hyper immune serum was raised against two isolates, namely, BGL and QS 1. Agar gel immunodiffusion carried out using both these hyper immune sera and whole cell protein of the isolates revealed clear lines of identity, indicating high degree of antigenic homogeneity in between the local isolates and also between the local isolates and the reference isolate.

The present study reveals high degree of antigenic homogeneity in between the local isolates and also between the local isolates and the reference isolate. Even though antigenic homogeneity existed between the reference isolate and local isolates, the reference isolate was comparatively less pathogenic. These findings may help in evolving a better vaccine against FT incorporating locally prevalent strains of the organism.

Summary

6. SUMMARY

The present study was conducted to evaluate the pathogenicity and antigenic relationship of various *Salmonella* isolates from Kerala in comparison with a reference/known strain. Three local isolates *viz.*, PS 8, QS 1, X 4 and a reference isolate BGL were used. The important parameters studied were antibiogram, pathogenicty in mice, pathogenicity in day-old chicks and in 18 week old layers, through three different routes and re-isolation of salmonellae from cloacal swabs, eggs, post mortem samples, gross as well as histopathology, OMP profile by SDS-PAGE and serological analysis by AGID.

All isolates were sensitive to amoxy clav, cephalexin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin and tetracycline. All were resistant to penicillin-G, nalidixic acid, erythromycin, cloxacillin and clindamycin. Except isolate X 4, all were found resistant to co-trimoxazole.

Except for the reference strain BGL, all the other three isolates were found to be pathogenic to mice. Among them the most pathogenic was isolate PS 8. The reference strain BGL failed to produce any clinical signs or mortality in mice even after seven days post infection.

All the four isolates were found to be pathogenic to day-old chicks. Clinical signs and mortality were predominant and appeared early in IM route, followed by oral and IC routes. The major clinical signs observed were somnolence, poor growth rate, weakness, inappetance and whitish diarrhea. Isolates QS 1 and X 4 were the most pathogenic, causing 100 per cent mortality, while isolate BGL was least pathogenic. Survived chicks had considerable loss in weight gain and showed intermittent shedding of salmonellae through feces till the end of experimental period. All chicks died during experimental period gave positive *Salmonella* isolation from liver, spleen, heart blood, lungs, yolk and

ceca. The survived chicks were sacrificed at the end of experimental period and positive isolation was obtained only from ceca.

Regarding the pathogenicity in layers the clinical signs and mortality were predominant and appeared early in intravenous route, followed by oral and IC route inoculated birds. The major clinical signs observed were listlessness, inappetance, ruffled feathers, shrunken combs and greenish-yellow diarrhea with gradual weight loss. There was considerable reduction in egg production. Cloacal swabs examined on day three, seven, eleven, fifteen, twenty one and twenty eight revealed intermittent shedding of organisms by birds in most of the experimental groups. The most pathogenic strain was found to be QS 1, which was not shedding the organism at the highest rate. The survived birds were sacrificed at the end of experimental period and on attempting re-isolation, the highest number was obtained from liver and oviduct, followed by spleen, ovary and ceca with equal number of isolations.

The egg production was affected in all challenged birds and among them, most affected was intravenously challenged groups. No significant differences could be noticed between different routes of inoculation and re-isolation from egg in all the four isolates.

The re-isolates were confirmed as *S. gallinarum* by biochemical characterization. All the isolates tested were methyl red positive and were indole, Voges-Proskauer, urease, phenylalanine deaminase and citrate negative.

The gross and hisopathological changes observed in experimental infection with all isolates were the same. There was enlargement and necrosis of liver and spleen with congestion of heart and lungs. Day-old chicks in addition revealed omphalitis. Histopathological changes were that of congestion, necrosis and infiltration of inflammatory cells into the internal organs including sub mucosa of intestinal walls.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis analysis of OMPs of all four isolates were conducted to study the antigenic relationship among isolates. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis revealed 13 different polypeptide bands of molecular weight ranging between 110 to 18 kDa for all the four isolates, indicating high degree of antigenic homogeneity between the isolates.

Agar gel immunodiffusion was carried out with hyperimmune serum raised against two isolates *viz.,* BGL and QS 1 to analyze the homogeneity/ heterogeneity of bacterial isolates by serology. Agar gel immunodiffusion carried out using both these hyperimmune sera and whole cell protein of isolates revealed clear lines of identity, indicating high degree of antigenic homogeneity in between the local isolates and also between local isolates and the reference isolate.

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EVALUATION OF PATHOGENICITY AND ANTIGENIC RELATIONSHIP OF *Salmonella* ISOLATES FROM POULTRY

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ABSTRACT

In the present study the pathogenicity and the antigenic relationship of three local isolates of *S. gallinarum* was compared with that of a reference/known strain. The antigenic homogeneity/heterogeneity of these isolates was studied using SDS PAGE and AGID.

All the four isolates when subjected to antibiogram revealed similar pattern. The only difference was with isolate X 4 which was susceptible to co-trimoxazole while all the other three were resistant.

Except for the reference/known strain BGL, all the other three isolates were found pathogenic to mice.

All the four isolates were pathogenic to day-old chicks. Isolates QS 1 and X 4 were most pathogenic. Major clinical signs were somnolence, weakness, inappetance and whitish diarrhea and were more prominent in IM inoculated group. All chicks that died during the experimental period gave positive *Salmonella* isolation from liver, spleen, heart blood, lungs, yolk and ceca. At the end of experimental period survived birds were sacrificed and they gave positive isolation only from ceca.

All the four isolates were pathogenic to layers. The clinical signs observed were listlessness, inappetance, ruffled feathers, shrunken comb, greenish-yellow diarrhea with gradual weight loss and was more prominent in IV challenged group, followed by those inoculated by oral and IC routes. The most pathogenic strain was QS.1. Cloacal swabs examined for *Salmonella* revealed intermittent shedding in all groups. The highest re-isolation was obtained from liver. The egg production was affected in all test groups and was most severely affected in IV group. No significant difference was noticed between different routes of inoculation and among different isolates in re-isolation from egg.

The re-isolates were confirmed as *S. gallinarum* by standard biochemical reactions.

The gross and histopathological changes observed in experimental infection with all the isolates were the same. There was enlargement and necrosis of liver and spleen, with congestion of heart and lungs. Day-old chicks had omphalitis. Histopathologically there was congestion, necrosis and infiltration of inflammatory cells into the internal organs, including submucosa of intestine.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis analysis of the OMPs of all four isolates revealed 13 different polypeptide bands. These bands were of similar molecular weight in all the four isolates, indicating antigenic homogeneity.

Agar gel immunodiffusion carried out using hyper immune serum raised against isolates BGL and QS 1 revealed lines of identity between the four isolates, indicating antigenic homogeneity by serology.