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NUCLEIC ACID BASED DETECTION OF SALMONELLAE IN 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 **"NUCLEIC ACID BASED DETECTION OF SALMONELLAE IN POULTRY"** is a bonafide record of research work done by me during the course of research and that the 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 this thesis entitled **"NUCLEIC ACID BASED DETECTION OF SALMONELLAE IN POULTRY"** is a record of research work done independently by **M. Muthuramalingam** 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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Introduction

1. INTRODUCTION

The poultry industry in India has shown tremendous developments during the last few decades, solving the problems of unemployment and malnutrition to a great extent. Despite the advancements, this industry incurs losses due to infectious diseases, especially the dreadful bacterial diseases. The disease scenario in poultry has been complicated by factors such as emergence of new diseases, re-emergence of hitherto controlled diseases, disease outbreaks by new variants and resultant breakdown of immunity, poor vaccination strategy, reservoir and spread of pathogens by unnatural hosts.

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.

The salmonellosis caused by genus *Salmonella,* is the most common cause of food-borne infection in the world. Recently salmonellosis in poultry has drawn greater awareness and attention of scientists because of the public health significance.

The unique wide host range exhibited by *Salmonella* covering mammals including cold-blooded animals, birds and reptiles signify the role of this organism as a potential pathogen.

Conventional cultural methods for *Salmonella* detection involve the use of non-selective pre-enrichment, followed by selective enrichment and plating on to selective and differential media. Suspect colonies are usually confirmed of their identity based on biochemical or serological procedures and these elaborate tests require minimum of five to seven days time. The conventional biochemical and serological methods of identification of bacteria require isolation, identification

and maintenance of live bacteria and extraction/ detection of antigen/ enzymes/ metabolites in them. But the present day molecular methods of detection of pathogen in the clinical material overcome many of the elaborate time consuming procedures.

The search for alternative methods for early diagnosis of *Salmonella* infection have been focused on DNA based techniques, like DNA hybridization and polymerase chain reaction (PCR), taking the advantage of recent developments in molecular biology. The target DNA can be increased to a detectable level by the use of a complimentary bit of nucleic acid, and by PCR, which exponentially amplifies the target sequence of nucleic acid in two to three hours time. Detection of amplified product indicates the presence of original target nucleic acid and hence the original target organism.

The advantages of PCR in detection of pathogen in the sample have shown to be delimited by the presence of inhibitory substances of PCR in the sample. Use of non-selective and/or selective enrichment combined with PCR have been applied for detection of many bacterial pathogens. This will improve the sensitivity of the test by diluting the PCR inhibitory substances.

Current diagnosis of salmonellosis has been possible by employing PCR and its modifications, using primers, which can specifically amplify or detect *Salmonella* up to serovar level. This approach has also been extensively applied for the detection on *Salmonella* in the environment of poultry keeping and are found to be sensitive enough.

Incidences of salmonellosis are frequently reported in chicken and other birds of this State and the diagnosis have been based on the isolation and identification of the causative agent. These isolates were characterized employing molecular methods (Mary, 2000).

Considering the sensitivity and easiness of the molecular methods for detecting *Salmonella* in birds, poultry products and environment, PCR and its

modifications were tried as an adjunct to the cultural methods in the present study. The approaches included in the present study were,

1. Detection of salmonellae by Polymerase Chain Reaction in avian bio-materials

2. Isolation and identification of salmonellae from avian bio-materials

3. Differentiation of salmonellae based on molecular methods.

Review of Literature

2. REVIEW OF LITERATURE

2.1 ETIOLOGY AND NOMENCLATURE

Salmonella belongs to the family *Enterobacteriaceae,* non-sporing Gram-negative rods that do not have capsules, non-acid fast and facultatively anaerobic. Most serotypes are motile with peritrichous flagella, but *Salmonella gallinarum* and *Salmonella pullorum* are non-motile (Wray *et al.*, 1996).

Salmonellae are intracellular bacteria that invade the mucous membrane and are transmitted to humans mainly through meat, egg and poultry products (D'Aoust, 1991).

It was in 1885 that Daniel E. Salmon, US, Veterinary Surgeon discovered the first strain of *Salmonella.* Today there are more than 2500 different serotypes and most of these serotypes are human pathogens (Kwang *et al.*, 1996).

The genus *Salmonella* comprises of two species; (I) *S. enterica*, which is divided into six sub species: *S. enterica* subspecies *enterica* (1), *S. enterica* subspecies *salamae*(II) *S. enterica* subspecies *arizonae* (III a), *S. enterica* subspecies *diarizonae* (III b), *S. enterica* subspecies *houtenae* (IV) and *S. enterica* subspecies *indica* (VI); and (2) *S. bongori* (formerly called S. *enterica* subspecies *bongori* V). Species and subspecies can be distinguished on the basis of differential characters, and these through antigenic formulae, onto 2,501 serovars. Usually the 1,478 serovars that belong to the *enterica* species *enterica* subspecies (I) colonize the enteric tract of warm- blooded animals, while the other 1,023 serovars belonging to subspecies of the II. IIIa. IIIb, IV and VI and to species *S. bongori* are found in cold- blooded animals, birds and in the environment (Popoff, 2001).

2.2 INCIDENCE

2.2.1 Incidence of Global Avian Salmonellosis

The caeca have long been considered the primary source of *Salmonella* in the chicken (Fanelli *et al.*, 1971).

Higgins *et al.* (1982) studied the dissemination of *Salmonella* spp. in nine broiler farms and reported that the frequency of contamination of *Salmonella* in feed was lower than that of litter, through water and pen dust.

Soerjadi-Liem and Cumming (1984) conducted a survey on the broiler flocks in Australia and reported that *Salmonella* spp. were isolated from flocks reared on new litter than on old litter. The five different *Salmonella* serotypes isolated were *S. eimsbeutel*, *S. havana*, *S. luonshya*, *S. singapore* and *S. typhimurium.*

Food poisoning outbreaks in general and those of *Salmonella enteritidis* in particular, have been associated with the consumption of infected poultry meat and egg products. The spread of *Salmonella enteritidis* to internal tissues of birds indicated systemic infection and suggested that transovarian transmission of eggs might occur (Rampling *et al.*, 1989).

The incidence of *Salmonella* on broiler carcasses had been shown to increase with successive stages of processing, possibly due to the ability of the *Salmonella* to live firmly attached to poultry tissues (Lillard, 1989).

Timoney *et al.* (1989) reported that isolation of *Salmonella* spp. from eggs might be due to the contamination of the egg contents by the invasion of the organism through the eggshell from faeces of hens excreting *Salmonella.*

In a study on *Salmonella* contamination in broiler flocks, *Salmonella* spp. were isolated from 108 of 875 (12.3 per cent) water samples and one or more

water samples from 63 of 292 (21.6 per cent) flocks were positive for *Salmonella* (Poppe *et al.*, 1992).

Many of the human *Salmonella enteritidis* infections had been traced to contaminated eggs and of those laying hens at the farm (Henzler *et al.*, 1994). People become infected with *Salmonella enteritidis* because of contacts with infected broiler breeder flocks, broiler rearing flocks and handling of infected broilers at slaughter (Corkish *et al.*, 1994).

Cox (1995) reported that an increase in human infections involving *Salmonella* had not been linked to consumption of any particular foodstuff, including eggs.

Hargis *et al.* (1995) evaluated gastro-intestinal contents as a source of *Salmonella* contamination for broiler carcasses and isolated *Salmonella* spp. from meat and reported that the broiler carcasses might be contaminated by gastro-intestinal contents during slaughtering process.

Broiler carcasses become contaminated by crop contents during the slaughtering process (Hargis *et al.*, 1995).

Salmonella enteritidis was isolated from 51 per cent of raw chicken and 23 per cent giblet samples during a study on *Salmonella* contamination of retail chicken products sold in the United Kingdom (Plummer *et al.*, 1995).

Caecal contents of 2,345 broiler chickens from 28 flocks of 12 farms were examined for the prevalence of *Salmonella* infection status. *Salmonella* spp. were isolated from 336 (14.3 per cent) of samples collected. *S. typhimurium* and *S. enteritidis* were detected in pooled broken egg samples collected from a hatchery. Environmental samples such as litter and water contained 78 and 55.6 per cent of *Salmonella* spp. respectively, whereas no organism was detected from feed samples (Limawongpranee *et al.*, 1999).

Soumet *et al.* (1999) reported that *Salmonella* were the most important pathogen of food-borne illness in France, leading to many hospitalizations and a few deaths each year. *Salmonella enterica* were mainly transmitted to humans following the consumption of contaminated eggs and poultry products.

Kumari *et al.* (2001) aimed to detect the prevalence of *Salmonella* spp. from apparently healthy quails and they isolated five *Salmonella gallinarum* and one *Salmonella worthington.*

Liebana *et al.* (2002) reported that S. Enteritidis and S. Typhimurium were the prevalent zoonotic serovars found in both breeding and commercial flocks of the broiler sector of the poultry industry confirming poultry meat to be one of the main sources of *Salmonella* infections in humans.

Milko *et al.* (2002) reported that one of the rare serovar of *Salmonella,* S. Paratyphi B has now been increasingly isolated from poultry and poultry products. These were also virulent pathogens to humans.

Roy *et al.* (2002) isolated *Salmonella* from 11.99 per cent of 4745 samples analysed. The samples comprised of meat, carcass rinse water, fluff samples, drag swabs and necropsy cases.

2.2.2 Incidence in India

Tucker (1967) isolated *Salmonella* spp. from fresh litter and reported that *Salmonella* serovars persisted longer in fresh litter than in built up litter samples in 55 per cent of broiler chicken houses studied. The *Salmonella* serovars isolated were *S. typhimurium, S. infantis, S. gallinarum* and *S. pullorum.*

Janakiraman and Rajendran (1974) reported the incidence of *Salmonella* in poultry carcasses (1.38 per cent), liver and spleen (1.7 per cent), eggshell wash (5.55 per cent), cloacal swabs/ intestinal contents (1.19 per cent) heart blood swabs (0 per cent), ovaries (0 per cent), abdominal egg mass (0 per cent) and unabsorbed yolk (0 per cent).

Bhatia and McNabb (1980) conducted a study on dissemination of *Salmonella* in broiler and processing plants. *Salmonella* spp. were isolated from litter, carcass washings, meconium, feed and fluff samples. The different *Salmonella* serotypes isolated were S. *typhimurium, S. infantis and S. schwarzengrund.*

Kapoor *et al.* (1980) reported that the Indian poultry industry had a great economic setback due to frequent occurrence of salmonellosis and presence of *Salmonella* in poultry and poultry products posed a great hazard to public health.

Palaniswami *et al.* (1989) recorded an incidence of *S. typhimurium* from a poultry farm in Mohanur, Tamil Nadu. The organism was isolated from heart blood swabs and bile swabs collected from the day- old dead chicks.

Salmonellosis is a hyper endemic disease in India affecting both man and animals alike. There are over 2464 serovars of *Salmonella,* distributed widely in nature, and in India, more than 235 serovars have been recorded, and this number is increasing constantly (Reeves *et al.*, 1989).

Purushothaman *et al.* (1996) reported that *Salmonella typhimurium* was the commonest serotype isolated from poultry and its environment and 34 *Salmonella* serotypes were isolated.

S. gallinarum and *S. enteritidis* were isolated from 39 poultry flocks from different parts of Haryana in India (Jindal *et al.,* 1999).

Saikia *et al.* (2002) have carried out isolation and antibiogram of *Salmonella* from enteric infection in man and animals and they found that S. Entertidis was the predominant serovar in poultry.

Batabyal *et al.* (2002) isolated S. Gallinarum from liver and intestine and characterized them biochemically and serologically. Seven per cent of the postmortem samples analysed were S. Gallinarum.

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

A total of 23 *Salmonella* isolates were recovered from 40 different samples collected from different disease outbreaks. Of these, 15,five and three isolates were recovered from samples of Karnataka, Maharastra and Tamil Nadu respectively. The serotype isolated were *Salmonella* Gallinarum, *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Worthington. The most predominant serotype was *S.* Gallinarum accounting for 69.6 per cent followed by *S.* Enteritidis for 21.7 per cent (Prakash *et al.*, 2005)

2.3 ISOLATION OF *SALMONELLA*

2.3.1 Samples

Fanelli *et al.* (1971) found caecal contents to provide the best evidence of *Salmonella.* But Smith *et al.* (1972) isolated *Salmonella typhimurium* more often from the Bursa of Fabricius than from the caecal contents.

Smith *et al.* (1972) used cloacal swabs for identifying *Salmonella* in experimentally infected chickens. By culturing eggshells and egg contents from experimentally infected laying hens, Cox *et al.* (1973) isolated very few *Salmonella.*

When cloacal tissues were heavily contaminated with *Salmonella enteritidis*, the eggs were culture positive, whereas, if these tissues had a low rate of infection, the eggs were culture negative (Keller *et al.*, 1995).

Purushothaman *et al.* (1996) reported that samples of materials originating from cloacal swabs, poultry feeds, poultry litter, fishmeal and caecal swabs formed the source for the isolation of *Salmonella.*

Skov *et al.* (1999) compared four different sampling methods potentially applicable for detection of *Salmonella* in broiler flocks, based on collection of faecal samples (i) by hand, 300 fresh faecal samples (ii) absorbed on five sheets of paper (iii) absorbed on five pairs of socks (elastic cotton tubes pulled over the boots and termed 'socks') and (iv) by using only one pair of socks. Their results showed that the sock method had sensitivity comparable with the hand collection method; the paper collection method was inferior, as was the use of only one pair of socks.

2.3.2 Pre-enrichment of Inoculum

Thomason and Dodd (1978) found advantage in using lactose broth for pre-enrichment over direct enrichment broth for recovering *Salmonella* from meat, poultry and environmental samples.

Cox *et al.* (1980) also observed that increased recovery from lactose pre-enrichment broth over direct enrichment broth, when examining meat or poultry samples.

Purushothaman *et al.* (1996) proposed the pre-enrichment with buffered peptone water for recovering *Salmonella* from meat, poultry, and environmental samples.

2.3.3 Incubation at Elevated Temperature

2.3.3.1 Use of Selective Media

Mac Conkey (1908) noted that incubation of the bile salt lactose agar at 42°C increased the selectivity of the medium. At the temperatures lower than 40°C problems of overgrowth by contaminating organisms occurred.

Livingston (1965) found that incubation of Salmonella-Shigella agar plates (SS agar) at 40°C allowed *Salmonella* to form colonies but controlled the

growth of unwanted organisms. The temperature was critical and some *Salmonella* strains, notably *Salmonella typhi* grew very well at 41°C.

Waltman *et al.* (1993) studied the optimum incubation times for *Salmonella* enrichment cultures. The results showed a step-wise increase in incubation would increase the *Salmonella* isolations.

2.3.3.2 Use of Enrichment Broth

Carlson *et al.* (1969) found a definite advantage in incubating broth at 43°C and plating after 48 hours for isolating *Salmonella* from poultry.

Elevated temperature incubation was effectively used for pre-enrichment in heart infusion broth. Chau and Huang (1976) also used incubation temperature above 37°C to increase the specificity for rapid detection of *Salmonella* in clinical specimens.

2.3.4 Enrichment Broth

2.3.4.1 Pre-enrichment Broth

Thomason *et al.* (1977) studied the recovery of salmonellae from environmental samples enriched with buffered peptone water (BPW) and reported that BPW was better than lactose broth for the isolation of *Salmonella.*

Buffered peptone water was used as the pre-enrichment medium for the isolation of *Salmonella* spp. from two groups of 100 chicks that were experimentally infected (Skov *et al.*, 2002).

2.3.4.2 Selenite Enrichment

Selenite media had one particular disadvantage. When sub cultured to bismuth sulphite agar, an area of growth inhibition appeared where the original inoculum was placed on the surface of the solid medium. This phenomenon had been discussed by Corry *et al.* (1969). The combined inhibitory effect can

prevent *Salmonella* isolation. It can be diminished by diluting the inoculum from selenite broth or by allowing the inoculum to dry on the bismuth sulphite plate before spreading.

Greenfield and Bigland (1970) studied the effect of selenite F broth on several *Salmonella*. *Salmonella typhi* did not grow well, when incubated at 43°C.

Selenite brilliant green enrichment was unsuitable for isolating *Salmonella dublin* (Harvey and Price, 1975). Selenite F broth is still one of the best enrichment media for the isolation of *Salmonella typhi*. Certain modifications, however, had been more successful than the original medium.

Selenite cystine was found to be the preferred medium for *Salmonella* isolation from broiler carcasses (Cox *et al.*, 1978). Purushothaman *et al.* (1996) proposed the selenite F selective enrichment broth for recovering *Salmonella* from meat, poultry and environmental samples.

2.3.4.3 Tetrathionate Enrichment Broth

Banwart and Ayrs (1953) found definite inhibition of *Salmonella paratyphi* by tetrathionate broth. To increase the selectivity, other workers had further modified tetrathionate broth by addition of novobiocin (Jefrries, 1959) and sodium lauryl sulphate (Jameson, 1961).

Media containing either selenite or tetrathionate plus brilliant green and bile or else malachite green in combination with high amounts of magnesium chloride are at present the predominant tools for selective enrichment (Busse, 1995).

2.3.4.4 Rappaport Vassiliadis (RV) Enrichment Broth

Iveson *et al.* (1964) obtained good results with Rappaport's enrichment medium for *Salmonella* isolation. Iveson and Kovacs (1967) showed that Rappaport's enrichment medium was superior to selenite F and tetrathionate broths. It appears to have a bias towards isolation of *Salmonella paratyphi* B.

Vassiliadis *et al.* (1983) recommended incubation of RV at 43°C. This achieved maximum selectivity, but any deviation above 43°C may be lethal for *Salmonella.*

Rappaport –vassiliadis medium had been generally accepted as a tool beside selenite and tetrathionate broths. Out of 2000 samples of meat, meat products, pig faeces and sewage, 17 per cent were found positive with Muller-Kauffmann (MK) and 25 per cent with RV (Busse, 1995).

Four selective enrichment broths were compared for the detection of *Salmonella* spp. in naturally contaminated poultry products and the recovery of atypical *Salmonella* strains in suspensions of pure cultures. In analysis of 100 poultry samples, the sensitivities observed were 94.0 per cent for Muller-Kauffmann Tetrathionate-Brilliant Green (MKTBG), 97.6 per cent for Rappaport Vassiliadis (RV), 42.2 per cent for Selenite Cystine (SC) and 97.6 per cent for the new broth KIMAN (Whitley Impedance Broth supplemented with 20 mg/ l of novobiocin sodium salt, 10 mg/ l of malachite green oxalate and 40 g/ l of potassium iodide). The two most efficient broths RV and KIMAN for recovery of atypical *Salmonella* strains were less toxic than MKTBG but more toxic than SC broth (Blivet *et al.,* 1997).

Hammack *et al.* (1999) studied the relative effectiveness of Selenite Cystine broth, Tetrathionate (TT) broth and RV medium for the recovery of *Salmonella* from food. It was reported that RV medium could be used for the analysis of food with high and low microbial load.

Employing the modified semi-solid Rappaport–Vassiliadis medium (MSRV), presumptive results for *Salmonella* can be obtained in 48 h, representing an interesting alternative to the standard methods (Barnika *et al.*, 2001).

Oliveira *et al.* (2002) used RV medium and TT medium as enrichment media for isolation of *Salmonella* spp. from 103 poultry derived field samples consisting of drag swabs, viscera, meconium and meat meal.

2.3.4.5 Enrichment with Antibiotic Supplement

Tate *et al.* (1990) showed that delayed secondary enrichment and enteric plates supplemented with novobiocin significantly improved *Salmonella* detection from the farm environmental samples.

A conventional method of isolating *Salmonella* was compared with isolation using novobiocin-supplemented plating media and delayed secondary enrichment (DSE). In comparison, a total of 421 (91 per cent) *Salmonella* were isolated by DSE, of which 195 isolates (42 per cent) were isolated only with DSE. The addition of novobiocin to the selective plating medium increased the isolation rate for *Salmonella* and reduced the level of contaminating bacteria growing on the plate (Waltman *et al.*, 1991).

2.3.5 Plating Media Used for Isolation

2.3.5.1 Brilliant Green Agar (BGA)

Corry *et al.* (1969), in a study of interactions in the recovery of *Salmonella typhimurium* damaged by heat or gamma radiation, found that BGA or Brilliant Green Phenolred agar were the most generally satisfactory plating media for the isolation of *Salmonella* along with tetrathionate enrichment.

On BGA *Salmonella* were identified by their inability to ferment lactose or lactose and sucrose, giving pink-colored colonies (Busse, 1995).

Purushothaman *et al.* (1996) proposed BGA for recovering *Salmonella* from meat, poultry and environmental samples.

2.3.5.2 Xylose Lysine Desoxycholate Agar (XLD)

Morinigo *et al.* (1986) used XLD as the plating media to isolate *Salmonella* organisms during a comparative study of seven enrichment media for detecting *Salmonella* from polluted freshwater.

Tate *et al.* (1990) stated that the more a medium was designed to isolate wider varieties of pathogens, the more other competing bacteria were able to grow, resulting in decreased selectivity.

Xylose Lysine Tergitol (XLT) Agar was used to isolate *Salmonella* organisms from poultry, poultry products and poultry environment (Roy *et al.*, 2002).

2.3.5.3 Mac Conkey's Agar (MCA)

Mac Conkey (1905) described Mac Conkey's agar for the selective isolation of Gram-negative enteric bacteria.

Mac Conkey's agar, a bacteriological medium that selects for Gram-negative bacilli and differentiates lactose fermenters from nonfermenters of lactose (NFL). Bile salts selectively inhibit Gram-positive organisms. When Gram-negative bacilli ferment lactose, mixed acid byproducts are formed. These acids cause a localized decrease in pH, shown by neutral red, producing pink-red colonies. Some lactose fermenters, such as *Escherichia coli*, also produce a zone of precipitated bile salts around the colonies. NFL produces no color (Flournoy *et al.*, 1990)

Amavisit *et al.* (2001) used Mac Conkey's agar as a direct plating medium to isolate *Salmonella* organisms from faecal samples of horse.

2.3.5.4 Hektoen Enteric Agar (HEA)

Stone *et al.* (1994) used Hektoen enteric agar for *Salmonella* isolation from chicken.

Agarwal and Bhilegaonbar (2001) developed a new Thin Agar Layer (TAL) method for the recovery of heat injured *S. typhimurium* and compared it with a two step overlay (OV) method by using four different selective media namely Bismuth Sulphite Agar (BSA), BGA, HEA and XLD Agar. Their result showed that all the four media BSA, BGA, XLD and HEA in TAL and OV methods were capable of recovering heat injured *S. typhimurium*. However, overall, HEA and BSA were found more efficacious than BGA and XLD.

2.4 IDENTIFICATION

2.4.1 Identification by Cultural Characters

Colonies on *Salmonella* on MCA are moderately large, thick, pale or colorless, moist circular disks; dome shaped and smooth, the opacity and size vary with different strains (Cruickshank *et al.*, 1972).

All *Salmonella* spp. failed to ferment either lactose and sucrose and their colonies appeared pink to red with reddening of media on BGA (Chakraborthy *et al.*, 1999).

Roy *et al.* (2002) observed *Salmonella* organisms as black colonies or red colonies with black centre on XLT agar.

2.4.2 Identification by Biochemical Characters

Millemann *et al.* (1995) observed differences in utilization of citrate and in the fermentation and oxidation of inositol, sorbitol and utilization of amygdalin among *Salmonella* species.

Purushothaman *et al.* (1996) confirmed the *Salmonella* isolates with triple sugar iron agar, lysine iron agar and other sugar fermentation patterns and reported that there was no significant relationship between inositol negative strains and multiple antibiotic resistance. They further inferred variation in the biochemical properties within *Salmonella* serovars.

Chakraborthy *et al.* (1999) described that *Salmonella gallinarum* fermented dextrose, mannitol, maltose, dulcitol and sorbitol with gas production and did not ferment adonitol, lactose, sucrose and salicin.

Lee *et al.* (2003) observed same pattern in the majority of biochemical properties such as IMViC, carbohydrate fermentation and amino acid decarboxylation. But, dulcitol and trehalose were fermented by 95.4 per cent and 93.7 per cent isolates and lysine and ornithine were decarboxylated by 99.8 per cent and 1.4 per cent respectively. Also sorbitol fermentation (59.1 per cent) and arginine decarboxylation (27.0 per cent) showed the diversity by isolates especially sorbitol fermentation increased annually from 11.1 per cent in 1995 to 82.6 per cent in 2001.

2.5 SEROTYPING

Blaxland *et al.* (1958) described the preparation and use of a stained whole blood *S. typhimurium* antigen for testing chickens, turkeys and ducks by a rapid macroscopic plate method. The sensitivity of the test was low.

Purushothaman *et al.* (1996) reported that the serotyping was least sensitive and more difficult to perform since to maintain sera for all the somatic and flagellar phases was difficult.

Rapid slide agglutination (RSA) test was done to serotype *Salmonella* isolates with the salmonella polyvalent somatic(O) antiserum and flagellar (H) antiserum according to the Kauffman-White Scheme (Roy *et al.*,2002).

Proux *et al.* (2002) aimed to detect the specific *Salmonella* serovar Gallinarum, which was divided into the biovars Pullorum and Gallinarum. Since the RSA test is based only on antigens from standard and variant strains of *S. pullorum*, it may not readily detect *S. gallinarum*. In their study, they detected infection in all 10-week-old chickens inoculated with *S. pullorum* strains but did not detect any antibodies against *S. gallinarum*. Therefore, *S. gallinarum* antigens must be added to the *S. pullorum* antigens used in the RSA test in order to detect antibodies produced by birds infected with either biovar.

Although conventional Kauffman-White Scheme is still the only reliable method for serotyping of *Salmonella*, however it could not differentiate between closely related biotypes *S. gallinarum* and *S. pullorum* (Shah *et al.*, 2005).

2.6 ANTIBIOGRAM

Lakhotia and Stephens (1973) reported that the resistance among *Salmonella* isolates to dihydrostreptomycin was most common (82.9 per cent), followed by resistance to tetracyclines (61.7 per cent), neomycin (36.1 per cent), triple sulfa (23.4 per cent) and colistin (2.1 per cent) and none of the *Salmonella* isolates were resistant to furazolidone, gentamicin, carbenicillin or nalidixic acid.

Cox *et al.* (1973) reported the acquisition of multiple drug resistance among *Salmonella* species. Yadav *et al.* (1985) reported that the antibiogram of the *Salmonella* isolates of different serotypes showed different patterns and such differences in antibiotic pattern indicated the epidemiological complication of salmonellosis.

Gast and Stephens (1985) provided evidence of a direct relationship between experimental antibiotic administration to turkeys and increased frequency of transfer of drug resistance plasmids to drug sensitive *Salmonella*.

Bokanyi *et al.* (1990) reported that *Salmonella* isolates revealed varying degree of resistance to triple sulfa, tetracycline, streptomycin, nalidixic acid, gentamicin, kanamicin and ampicillin.

Cox *et al.* (1996) reported antibiotic sensitivity of *Salmonella* strains to nitrofurantoin. Thirty nine per cent were sensitive, 53.0 per cent were intermediate and only 8.0 per cent were resistant, and for furazolidone 64.0 per cent were sensitive, 18.0 per cent were intermediate and 18.0 per cent were resistant.

Purushothaman *et al.* (1996) observed the resistance to chloramphenicol (4.62 per cent), doxycycline (73.2 per cent) erythromycin (64.62 per cent), chlortetracycline (53.85 per cent) and polymyxin B (46.15 per cent) among the *Salmonella* isolates.

Shivhare *et al.* (2000) reported that ciprofloxacin, enrofloxacin and sparfloxacin were found effective against all the *Salmonella* isolates. The per cent sensitivity of strains towards other antibiotics was 92 per cent for norfloxacin, 88 per cent for refloxacin, 84 per cent each for cefuroxime and cefroperazone, 44 per cent for aphlexin, 32 per cent for gentamicin, 16 per cent each for ampicillin, chloramphenicol and doxycycline and 8 per cent for cloxacillin. All the isolates were resistant to carbencillin, piperacillin, furazolidone, sulphonamides and trimethoprim.

Shah *et al.* (2001) characterized the *S. gallinarum* strains on the basis of their biotype, drug resistance and possible virulence associated factors like bacteriocin production and serum resistance.

Saikia *et al.* (2002) found that most of the strains of *Salmonella* were sensitive to enrofloxacin, chloramphenicol, gentamicin, nalidixic acid and norfloxacin. The isolates were resistant to ampicillin-cloxacillin combination, amoxycillin, furazolidone and tetracycline.

Lee *et al.* (2003) reported that the majority of *Salmonella gallinarum* isolates were susceptible to amoxycillin/clavulanic acid (92.3 to 100 per cent) colistin (97 per cent to 100 per cent) and sulfamethoxazole-trimethoprim (92.3 to 100 per cent). The prevalence of completely resistant isolates against antimicrobial drugs increased from 90 per cent in 1995 to 93.5 per cent in 2001.

Sujata *et al.* (2003) carried out antigenic characterization and antibiotic sensitivity of field isolates of *Salmonella gallinarum* and they found that all the isolates were 100 per cent sensitive to ciprofloxacin, amoxycillin, chloramphenicol and ampicillin.

Saxena *et al.* (2005) examined 24 *Salmonella* strains for their drug resistance. Strains lacking the plasmid also showed resistance for many antibiotics including ciprofloxacin, lincomycin, tetracycline, nitrofurazolidone and erythromycin.

2.7 PLASMID PROFILES OF *SALMONELLA*

The plasmids ranging in molecular weight from 300 kilobase (kb) to 2 kb were isolated from various *Salmonella* serovars. In *Salmonella typhimurium*, 60 Megadalton (Mdal) plasmid was reported by Helmuth *et al.* (1985). Plasmid profiling for differentiation of bacterial strains usually depends on the number of plasmids present in each strain.

Ikeda and Hirsh (1985) cautioned that the plasmid profile might not be a reliable method for bacterial subtyping because of the possibility of bacteria loosing or acquiring a new plasmid.

Helmuth *et al.* (1985) observed that 90.0 per cent or more of *Salmonella typhimurium* and *Salmonella enteritidis* isolates carried serotype specific plasmids with molecular masses of 60 Mdal and 37 Mdal respectively.

Nakamura *et al.* (1986) demonstrated that 37 Mdal plasmid was harboured by *Salmonella enteritidis* to encode some virulent functions and found

that large molecular mass plasmids were required for the virulence of several serotypes of *Salmonella*, which included *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella paratyphi* C .

There was a great deal of evidence that plasmid-linked genes contribute to the virulence of several *Salmonella* serotypes, which produced systemic disease in animals and humans. Such serotypes included *Salmonella typhimurium*, *S. dublin*, *S. enteritidis*, *S. cholerae suis*, *S. paratyphi* and *S. abortus ovis*. Elimination of the large plasmids from *S. gallinarum* and *S. pullorum* produced strains with significantly reduced virulence for chickens. Re-introduction of the plasmids restored full virulence. The plasmids were also responsible for the ability of the strains to survive and multiply in the cells of the reticuloendothelial system. (Barrow and Lovell, 1989).

The size of plasmids isolated from strains of *Salmonella* ranged from less than 1 Mdal to 180 Mdal (Threlfall and Frost, 1990). Baggesen *et al.* (1992) demonstrated a 90 kb plasmid in *Salmonella typhimurium* by plasmid profiling combined with restriction analysis.

Dorn *et al.* (1992) identified *Salmonella enteritidis* species-specific plasmid with the size of 38 Mdal (54 kb).

Olsen *et al.* (1994) reported that the way of cultures was treated prior to analysis might influence the plasmid profiles, because plasmid loss might occur in stab cultures during storage.

Rankin *et al.* (1995) reported that many isolates of *Salmonella enteritidis* did not have serotype specific plasmids.

Millemann *et al.* (1995) reported that 90 kb plasmid was found to be the serotype specific plasmid of *Salmonella typhimurium* and 54 kb plasmid was found to be the serotype specific plasmid of *Salmonella enteritidis*.

Puruthosthaman *et al.* (1996) reported the plasmids in the range of 45.71 kb to 26 kb in *Salmonella typhimurium* and also the non-plasmid containing *Salmonella typhimurium* showed resistance against some antibiotics.

Rathore *et al.* (1998) showed the presence of plasmid bands of 85 kb, 2 kb and 0.1 kb in *Salmonella typhimurium* and the plasmid yield was better in cultures kept at shaker incubator as compared to the stationary phase incubation.

Salmonella virulence plasmids are heterogeneous in size (50 to 90 kb), but all share a 7.8 kb region, *spv*, required for bacterial multiplication in the reticuloendothelial system. Other loci of the plasmid, such as the fimbrial operon *pef*, the conjugal transfer gene *traT* and the enigmatic *rck* and *rsk* loci, might play a role in other stages of the infection process. The virulence plasmid of *Salmonella typhimurium* LT2 is self-transmissible; virulence plasmids from other serovars, such as *Salmonella enteritidis* and *Salmonella choleraesuis*, carry incomplete *tra* operons. The presence of virulence plasmids in host-adapted serovars suggested that virulence plasmid acquisition might have expanded the host range of *Salmonella* (Rotger and Casadesus, 1999).

Rathore *et al.* (2000) found 0.1 kb, 2.0 kb, 85 kb, 85.2 kb and 100 kb plasmids in *Salmonella typhimurium*. Amplification of plasmids by addition of chloramphenicol (170 μg/ml) in the large-scale preparation resulted in higher yield of DNA.

Saxena *et al.* (2005) carried out plasmid profiling of 24 *Salmonella* isolates and a uniform plasmid profile was observed in 19 out of 24 strains. Six strains did not show the presence of any plasmid. In all other strains a single plasmid approximately of 85 kb was observed. The 85 kb plasmid revealed a positive correlation with virulence of *Salmonella* strains.

2.8 MOLECULAR METHODS FOR DETECTION OF *SALMONELLA*

In recent years, identification and characterization have favored analysis that reflects one of the most fundamental properties of an organism, its genetic information. Molecular approaches such as DNA hybridization and nucleic acid amplification have allowed bacterial detection directly from clinical samples, dramatically reducing the time required for identification.

2.8.1 Polymerase Chain Reaction

Saiki *et al.* (1988) reported that PCR was a sensitive and rapid technique and a few copies of target DNA could be amplified to a level detectable by gel electrophoresis or hybridization.

Li *et al.* (1988) reported that direct amplification of PCR to complex substrates resulted in no amplification products.

Most PCR based tests rely on DNA purification by proteinase K, phenol chloroform extraction and ethanol precipitation (Gouvea *et al.*, 1990).

Stone *et al.* (1994) also found that no amplification was observed on ethidium bromide staining after gel electrophoresis when the amount of DNA used in PCR amplification was as low as 30 pg of the total DNA.

The great advantage of the PCR procedure is that it can be applied to mixed microbial specimens without prior isolation of individual species of bacteria (Schrank *et al.*, 2001).

PCR is an enzymatic technique to amplify a specific DNA segment *in vitro* using two site-specific primers that hybridize to complimentary DNA strands. The method produces large amount of specific DNA from a complex DNA template in a single enzymatic reaction within a matter of hours (Sharma *et al.*, 2002).

Agarose gel electrophoresis followed by staining with ethidium bromide represents the simplest and most common method to analyse PCR product. PCR product is of a defined length. The size of PCR product may be calculated by simultaneous running of DNA molecular weight marker on the agarose gel (Sharma *et al.*, 2002).

2.8.2 PCR for Genus *Salmonella*

Molecular and functional characterization of the *Salmonella* invasion gene, *inv*A indicated that the *inv*A gene sequence was unique only to *Salmonella* and not in *Yersinia* spp., *Shigella* spp. and enteropathogenic *E.coli* (Galan *et al.*, 1992).

Direct PCR-based *Salmonella* detection in faecal specimens was hampered by inhibitory compounds, such as bilirubin and bile salts. These faecal compounds showed significant inhibition of PCR at low concentrations (10 to 50 micrograms/ml). So, faecal samples must be diluted 500 fold to overcome inhibition by the inhibitory compounds. Therefore, the magnetic immuno PCR assay (MIPA), which combines immunomagnetic separation by using specific monoclonal antibodies and PCR, was used directly to detect salmonellae in faeces from humans (Widjojoatmodjo *et al.*, 1992).

Fluit *et al.* (1993) described the magnetic immuno-polymerase chain reaction assay (MIPA). This MIPA performed with a *Salmonella* group B-specific monoclonal antibody and *Salmonella*-specific PCR primers based on the origin of DNA replication was capable of detecting 10 CFU of *Salmonella typhimurium* in the presence of 10^7 CFU of *Escherichia coli.* They showed that when the MIPA was used in combination with enrichment, as few as 0.1 CFU of *Salmonella* spp. per g of poultry meat could be detected reliably within 30 h.

Aabo *et al.* (1993) developed PCR primers for genus level detection of *Salmonella* spp. by selecting from a *Salmonella* specific fragment of 2.3 kb. The specific PCR product of 429 bp was found from 144 of 146 *Salmonella* strains

tested. No PCR product was produced in any of the 86 non-*Salmonella Enterobacteriaceae* strains tested covering 41 species from 21 genera.

The PCR technique had been applied to identify several bacterial specimens including *Salmonella* from food and clinical samples, using unique gene sequences as primers (Doran *et al.*, 1993).

Oligonucleotide primers for the PCR that enabled genus-specific detection of members of the genus *Salmonella* were developed. The primers amplified a 4976 bp genetic sequence of members of the genus *Salmonella* (Cohen *et al.*, 1994).

Stone *et al.* (1994) reported that the *Salmonella* species produced the PCR amplified DNA fragment of the size 457 bp and also found the false negative reactions.

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).

Luk *et al.* (1997) described a digoxigenin-based enzyme-linked immunosorbent assay (DIG-ELISA) following PCR to detect the amplified lipopolysaccharide *rfbS* gene as a means for rapid screening of serogroup D salmonellae in stool specimens. For pure bacterial cultures, the sensitivity of the PCR DIG-ELISA was approximately 10 bacteria. In the presence of stool materials, the salmonellae were first isolated by an immunomagnetic separation technique with an O9-specific monoclonal antibody, MATy-O9, followed by PCR and DIG-ELISA. The corresponding sensitivity was about 10 to 100 bacteria.

A commercial PCR based system BAX was evaluated to determine the efficiency of the system with different concentrations of *Salmonella* cells. A comparison with conventional bacterial culture method was made. The assay was able to detect 10^4 *Salmonella* CFU/ml of enrichment medium, which allowed consistent detection of *Salmonella* cells within 24 to 26 hours. The high degree of sensitivity and specificity of the BAX system made it a reliable alternative to conventional bacterial cultural methods. *Salmonella* cells were detected in 23 of 150 (15 per cent) processed chicken rinse samples with BAX system, compared to 18 of 150 (12 per cent) samples with conventional method (Bailey, 1998).

Rychlik *et al.* (1999) developed a simple and universal protocol for the rapid detection of *Salmonella* spp. in various samples using nested PCR. The detection limit for the nested PCR was 10^5 CFU/g of faeces even without pre-enrichment. After pre-enrichment it was possible to detect *Salmonella* in faeces with the original concentration of 10^2 CFU/g. In meat samples, it was possible to detect *Salmonella* spp. where the original concentration was less than 10 cells/g.

An immunoconcentration-PCR (ICS-PCR) assay was developed by Soumet *et al.* (1999) for the rapid and specific detection of *Salmonella.* This assay was evaluated against a conventional bacteriological method for the detection of *Salmonella* from environmental swabs of poultry houses. The 120 samples investigated were pre-enriched in phosphate buffered peptone water and *Salmonella* was separated by an immunoconcentration process using an automated system prior to PCR.The specificity of the assay was high as no false-positives were found. The sensitivity of the assay was 70 per cent. The correlation between the ICS-PCR assay and the bacteriological method was 84 per cent.

A simple and ready-to-go test based on a 5 nuclease (TaqMan) PCR technique was developed for identification of presumptive *Salmonella enterica* isolates. The results were compared with those of conventional methods. The PCR test correctly identified all the *Salmonella* strains by positive end-point

fluorescence (FAM) signals for the samples and positive control (Hoorfar *et al.*, 2000)

In comparison with conventional cultural methods, the IMS-PCR was a rapid, specific method for the detection of *Salmonella* in foods that contained neither fat nor a high amount of other microorganisms. Combined with the use of filtration bags, it reduced the negative effects of food matrix and gave the ability to detect *Salmonella* cells within 24 h (Jenikova *et al.*, 2000)

Carli *et al.* (2001) developed a rapid detection procedure for salmonellae from chicken faeces by the combination of tetrathionate primary enrichment-bacterial lysis-capillary PCR and capillary gel electrophoresis. These results indicated that TTB enrichment, bacterial lysis, and genus-specific capillary PCR combined with capillary gel electrophoresis constituted a sensitive and selective procedure, which had the potential to rapidly identify *Salmonella*-infected flocks.

A PCR method for the detection of *Salmonella* spp. in food was developed using *inv*A gene sequence as primers. The method was sensitive and specific and showed excellent correlation with the conventional method of reference when naturally contaminated foods were analyzed. Moreover, it was easily performed within a maximum of 12 h from food sampling, thus allowing prompt detection of *Salmonella* spp. in the food stocks analysed (Ferretti *et al.*, 2001).

The presence of *Salmonella* was assessed in 198 skin samples of poultry obtained from a commercial slaughter point by traditional culture method and by a *Salmonella* specific PCR test. *Salmonella* were recovered from 32 (16 per cent) of all samples tested using traditional culture methods. In contrast, PCR assay was proved to be more sensitive and detected *Salmonella* DNA in 38 (19 per cent) of the samples tested (Whyte *et al.*, 2002).

Trkov and Augustin, (2003) developed a molecular method for the detection of *Salmonella enterica* strains based on 16S rRNA sequence analysis.

Croci *et al.* (2004) compared an electrochemical enzyme-linked immunosorbent assay (ELISA) coupled with flow injection analysis (ELISA-FIA) and a PCR-based method using ST11 and ST15 primers, for detecting salmonellae in meat, and their results showed that both ELISA-FIA and PCR allowed detection of *Salmonella* in a product contaminated with a low number of the microorganisms (1 to 10 salmonellae/25 g) after only 5 h of incubation of pre-enrichment broth, and they were just as effective as the International Organization for Standardization (ISO) culture method.

Jin *et al.* (2004) concluded that, the two2-oxoglutarate dehydrogenase (OGDH) primers namely OGDH –1,OGDH-2 were found to be rapid and sensitive detectors of Salmonella spp for the PCR method.

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.

2.8.3 Serovar Specific *Salmonella* PCR

Arbitrarily primed PCR was a potential tool in ecological studies of food borne pathogens such as *Salmonella* (Fadl *et al.*, 1995). They concluded that an MK 22 primer in *Salmonella enteritidis* produced PCR products ranging from 0.65 kb to 1.7 kb. *Salmonella typhi* and *Salmonella typhimurium* produced 0.73 kb fragments on using the MK 22 primer.

Polymerase chain reaction with the specific gene sequence *sef*A encoded by all isolates of S. Enteritidis was developed to detect the organism in eggs (Woodward and Kirwan, 1996).

2.8.4 PCR for Virulence Plasmid

There are nearly 2200 *Salmonella* serovars, and all of those tested so far seem to contain *inv* genes, which enable the bacteria to invade cells. There are six *Salmonella* serovars known to contain the virulence plasmid carrying *spvC* genes: *S. typhimurium*, *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum* and *S.pullorum*. Except for *S. gallinarum* and *S. pullorum*, which are specific for fowl, the other serovars named here are common etiologic agents of enteritis in humans. Therefore, the appearance of at least one band, or two bands if there was a virulence plasmid, would indicate the presence of *Salmonella* (Jenikova *et al.*, 2000).

Amplified products were detected by one per cent agarose gel electrophoresis pre-stained with ethidium bromide, at 100 V for one hour. A positive result (the *Salmonella* specific band) was indicated by a fluorescent band at the 244 bp level. If the target *Salmonella* had the virulence plasmid, a second band was detected at the 571 bp level (Jenikova *et al.*, 2000).

2.8.5 PCR with Enrichment Broth

Gouws *et al.* (1998) reported a reliable *Salmonella* PCR detection method yielding results within 24 h. Artificially contaminated chicken samples were pre-enriched in BPW for 6 h. The DNA was extracted and using the *Salmonella* specific primers ST11 and ST15, a 429 bp PCR product was amplified. This PCR product was obtained in artificially contaminated samples with a detection limit of 50 CFU.

Schrank *et al.* (2001) coupled a culture procedure with PCR using the genus specific *inv*E *inv*A genes as primers. The expected 457 bp specific DNA fragment was amplified from dilutions containing as few as 5.7 CFU. This indicated that the PCR technique could be successfully coupled with culture in an enrichment broth to distinguish *Salmonella* spp. from other enteric bacteria

present in samples of poultry industry. The sensitivity and specificity of the PCR was more than that of culture method.

PCR specific assay was developed for the generic detection of *Salmonella* spp. in materials collected in the field from poultry. The specificity and sensitivity of the assay combined with RV selective enrichment broth (PCR – RV) were compared with standard culture method. An *inv*A gene specific oligonucloeotide primers were used to detect the generic *Salmonella.* The PCR amplification product was 284 bp. The PCR assay detected 128 per cent more positive samples than standard culture method. The sensitivity was determined by chi – square method (Oliveira *et al.*, 2002).

2.8.6 PCR- Restriction Fragment Length Polymorphism (PCR-RFLP)

Flagellin genes from 264 serovars of *Salmonella enterica* were amplified by two phase-specific PCR systems. Amplification products were subjected to restriction fragment length polymorphism (RFLP) analysis by using endonucleases *Hha* I and *Hph* I. RFLP with *Hha* I and *Hph* I yielded 64 and 42 different restriction profiles, respectively, among 329 flagellin genes coding for 26 antigens (Zabrovskaia *et al.*, 1998).

Lindstedt *et al.* (2000) performed the fluorescent labelled amplified-fragment length polymorphism (FAFLP) method on 97 strains of the food-borne pathogen *Salmonella enterica* subsp. *enterica* comprising seven different serovars, using the restriction enzymes *Eco*R I and *Mse* I. From the total FAFLP fingerprinted strains, 81 were compared with pulsed-field gel electrophoresis (PFGE) typing of the same strains. The FAFLP method showed a discriminatory power equal to that of PFGE.

PCR-restriction fragment length polymorphism (PCR-RFLP) and PCR–single-strand conformation polymorphism (PCR-SSCP) analyses were carried out on the 1.6 kb *groEL* gene from 41 strains of 10 different *Salmonella* serovars. Three *Hae* III RFLP profiles were recognized, but no discrimination between the

serovars could be achieved by this technique. However, PCR-SSCP analysis of the *groEL* genes of various *Salmonella* serovars produced 14 SSCP profiles, indicating the potential of this technique to differentiate different *Salmonella* serovars (Nair *et al.*, 2002).

Park *et al.* (*2001*) cloned and sequenced a fragment of *rfbS* gene and found polymorphic nucleotides specific for *salmonella gallinarum*(SG) and *salmonella pullorum*(SP). SG isolates had adenine and guanine residues at position 598 and 237 respectively, while SP had guanine and adenine residues at the respective positions. Since polymorphism in *rfbs* gene could be a good molecular marker, a PCR-RFLP method for differentiation of SG from SP was developed.

Park *et al.* (2001) first amplified the *rfbS* genes by PCR and then digested the amplified product with two restriction enymes namely *Tfi* I and *Ple* I. They found that DNA amplicon from *S. gallinarum* was given a digestion product of 235 bp with *Tfi* I but there was no digestion with *Ple* I. *S. pullorum* DNA amplicons gave a digestion product of 239bp with P*le* I. But was not digested by *Tfi* I.

2.8.7 Multiplex PCR (m-PCR)

Way *et al.* (1993) developed a m-PCR with three sets of primers, *pho*P, *Hin* and *H-li* which simultaneously detected the *Salmonella* spp., *S. typhi*, *S. typhimurium*, *S. paratyphi* A and *S. enteritidis* specifically.

Soumet *et al.* (1999) evaluated a m-PCR assay for simultaneous identification of *Salmonella* spp., *S. typhimurium* and *S. enteritidis* from environmental swabs of poultry houses. Three sets of primers were used for the assay. The ST11 - ST15 primers were specific for the genus *Salmonella*. The S1-S4 primers were selected from a gene associated with virulence and were specific for *S. enteritidis* and Fli 15-Typ 04 primers selected from *fli*C gene sequence were specific for *S. typhimurium*. The amplification product was 429

bp for genus level *Salmonella,* 559 bp for *S. enteritidis* and 312 bp for *S. typhimurium*.

Jeníkova *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.

The PCR primers for O, H, and Vi antigen genes, *tyv* (*rfbE*), *prt* (*rfbS*), *fliC-d*, *fliC-a*, and *viaB*, were designed and used for the rapid identification of *Salmonella enterica* serovars Typhi and Paratyphi A with multiplex PCR. The results showed that all the clinical isolates examined of *Salmonella* serovars Typhi and Paratyphi A were accurately identified by this assay. (Hirose *et al.*, 2002)

The polymerase chain reaction and the multiplex polymerase chain reaction were developed for detection of *Salmonella* and for identification of the serotype *enteritidis*. Three sets of primers were selected from different genomic sequences amplifying a 429 bp fragment specific for the genus *Salmonella* within a randomly cloned sequence, including a 250 bp fragment within the *spv* gene, and a 310 bp fragment within the *sef*A gene specific for *Salmonella enteritidis.* The *sef*A gene was detected in all 27 strains of *S. enteritidis* by this polymerase chain reaction method. Multiplex polymerase chain reaction could detect three genes in all strains, but could not detect the *spv* gene in two strains. (Pan and Liu, 2002).

A multiplex PCR method incorporating primers flanking three variable-number tandem repeat (VNTR) loci (arbitrarily labeled TR1, TR2, and TR3) in the CT18 strain of *Salmonella enterica* serovar Typhi was developed by Liu *et al.* (2003). They demonstrated that the multiplex PCR could be performed on crude cell lysates and that the VNTR banding profiles produced could be easily analyzed by visual inspection after conventional agarose gel electrophoresis.

2.7 GENE SEQUENCING

Six distinct clones were present among Greek multidrug-resistant *Salmonella enterica* serotype Typhimurium phage type DT104, since isolates belonging to resistance phenotypes including the ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) core could be distinguished with respect to their pulsed-field gel electrophoresis patterns, *int*1 integron structures, and presence or absence of antibiotic resistance genes *ant*-Ia, *pse*-1, and *tem*-1 (Markogiannakis *et al.,* 2000).

A DNA sequence was identified in isolates of *Salmonella enterica* serotype Typhimurium definitive type 104 (DT104). The PCR amplification of an internal segment of this sequence identified DT104 and the closely related U302 phage type among 146 isolates of *S. enterica* serotype Typhimurium tested, thus providing a tool for rapid identification of DT104 and related isolates (Pritchett *et al*., 2000).

Phylogenetic analysis of about 200 strains of *Salmonella*, *Shigella*, and *Escherichia coli* was carried out using the nucleotide sequence of the gene for DNA gyrase B (*gyrB*), which was determined by directly sequencing PCR fragments. The results established a new phylogenetic tree for the classification of *Salmonella*, *Shigella*, and *Escherichia coli* in which *Salmonella* forms a cluster separate from but closely related to *Shigella* and *E. coli.* In comparison with 16 S rRNA analysis, the *gyrB* sequences indicated a greater evolutionary divergence for the bacteria. Thus, in screening for the presence of bacteria, the *gyrB* gene might be a useful tool for differentiating between closely related species of bacteria such as *Shigella* spp. and *E. coli* (Fukushima *et al.*, 2002).

Kotetishvili *et al.* (2002) developed a multilocus sequence typing (MLST) based on the 16S RNA, *pduF*, *glnA*, and *manB* genes for *Salmonella*, and its discriminatory ability was compared to those of PFGE and serotyping. PFGE 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.

McQuiston *et al.* (2004) developed a system for determination of serotype in *Salmonella* based on DNA markers. To identify flagellar antigen-specific sequences, they have sequenced 280 alleles of the three genes that are known to encode flagellin in *Salmonella*, *fliC*, *fljB*, and *flpA*, representing 67 flagellar antigen types. Substantial sequence heterogeneity existed between alleles encoding different flagellar antigens while alleles encoding the same flagellar antigen were homologous, suggesting that flagellin genes might be useful targets for the molecular determination of flagellar antigen type.

Tanaka *et al.* (2004) carried out molecular characterization of a prophage of *Salmonella enterica* Serotype Typhimurium DT104 and they found that the entire DNA sequence consisted of 41,391 bp, including 64 open reading frames, and exhibited high similarity to P22 and to phage type conversion phage ST64T.

Materials and Methods

3. MATERIALS AND METHODS

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

3.1 ISOLATION OF *SALMONELLA*

3.1.1 Materials

3.1.1.1 Clinical Samples

Clinical samples for the detection and isolation of *Salmonella* were collected from ailing/dead birds brought to the Department of Microbiology and Pathology, College of Veterinary and Animal Sciences, Mannuthy, for disease diagnosis and from sick/ dead birds of University Poultry and Duck Farm, Mannuthy. Samples from healthy birds and environmental samples were collected from the following places: 1.Government Poultry Farm, Koovappadi, Kerala 2. Government Poultry Farm, Chathamangalam, Kerala. Cloacal swabs were collected from healthy and ailing/dead birds. From sacrificed ailing/dead birds tissues such as liver, spleen, ovary and caecal tonsils were collected.

One hundred and fifty five birds were screened for *Salmonella* including 119 chicken, 5 ducks, 19 quails and 12 turkeys. A total of 296 samples (cloacal swab, liver, spleen, ovary, caecal tonsils, intestine) collected from the above 155 birds were used for *Salmonella* isolation (Table 1).

Table 1. Details of clinical materials used for isolation of *Salmonella*

Sl. No.	**Nature of material**	**No. of samples examined**
1	Cloacal swab	155
2	Liver	51
3	Spleen	51
4	Ovary	6
5	Caecal tonsils	8
6	Intestine	25
	Total	296

Thirty-four environmental samples were also screened for *Salmonella*. (Table 2)

Table 2. Details of materials of environmental origin used for isolation of *Salmonella*

Sl. No.	Nature of material	No. of samples examined
1	Hatcher tray swab	10
2	Droppings	4
3	Drag swab	4
4	Feed	4
5	Water	4
6	Setter tray swab	4
7	Egg surface swab	4
	Total	34

3.1.1.2 Transport Media

Buffered peptone water (BPW) with antibiotic novobiocin at the rate of 0.5 μg/milliliter was used as a transport medium. This medium procured from Hi-media and was used as per manufacturer's instruction.

3.1.1.3 Media for Isolation of Salmonella

RV broth, BGA, 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 .

3.1.2 Method

3.1.2.1 Isolation of Salmonella

For isolation of *Salmonella* spp. from poultry, cloacal swabs, tissues and environment samples were taken. From the dead/ailing bird samples like liver, spleen, ovary, caecal tonsil and intestine were directly plated on to MCA and BGA and cloacal swabs were collected in BPW.

3.1.2.2 Resuscitate in Non- selective Pre-enrichment Broths

Samples like droppings, poultry feed and intestinal contents were added to BPW in the ratio of 1:10 (one part of sample and nine parts of broth). Samples like cloacal swab and egg surface swab were directly taken in BPW. The above samples were incubated at 37°C for one day.

3.1.2.3 Selective Enrichment

From the above inoculated, incubated BPW one-milliliter broth was taken, inoculated in to RV broth and further incubated at 42°C for 48h. From that two milliliter of broth was taken and used for PCR analysis (Table 3).

Table 3. Details of materials used for Rappaport-Vassiliadis - Polymerase Chain Reaction (RV-PCR) assay

Sl. No.	Nature of material	No.of samples examined
1	Cloacal swab	155
2	Intestine	25
3	Hatcher tray swab	10
4	Droppings	4
5	Drag swab	4
6	Water	4
7	Setter tray swab	4
8	Egg surface swab	4
	Total	210

Same time direct plating on to selective media like MCA as well as BGA was also done. Gram-negative non-lactose fermenters presented in MCA and pink colonies obtained in BGA were selected, sub cultured to obtain pure culture for further identification.

3.2 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, and production of acid from carbohydrates (glucose, cellobiose, maltose, mannitol, sucrose, dulcitol, sorbitol, trehalose, xylose and arabinose), as described by Barrow and Feltham (1994).

3.3 ANTIBIOGRAM

3.3.1 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.	Ampicillin(A)	10 µg
2.	Cloxacillin (Cx)	5 µg
3.	Amoxycillin (Ax)	10 units
4.	Enrofloxacin (Ex)	10 µg
5.	Pefloxacin (Pf)	5 µg
6.	Gentamicin (G)	30 µg
7.	Tetracycline (T)	10 µg
8.	Furazolidone (Fr)	100 µg
9.	Chloramphenicol (C)	3 µg
10.	Co-trimoxazole (Co)	25 µg
11.	Ciprofloxacin (Cf)	10 µg

3.3.2 Method

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

3.4 SEROTYPING

The isolates were sent to National Salmonella and Escherichia Center, Kasauli, Himachal pradesh, for serotyping.

3.5 AMPLIFICATION OF NUCLEIC ACID

3.5.1 Preparation of Template DNA

3.5.1.1 Materials

3.5.1.1a Phosphate Buffered Saline (PBS, 10 X)

NaCl	80.00 g
KCl	2.00 g
Na_2HPO_4	11.33 g
KH_2PO_4	2.00 g

Distilled water (DW) to 1000 ml.

The pH was adjusted to 7.2 and autoclaved at 121°C for 15 min at 15 lbs pressure and stored at room temperature.

3.5.1.1b PBS(1X)

PBS 10 X	100 ml
DW	900 ml

3.5.1.2 Method

3.5.1.2a Preparation of Template DNA from RV Broth Enriched Sample

From the RV broth, which was inoculated and incubated for two days, two milliliter was taken and transferred in to an Eppendorf tube and centrifuged at 3000x g for 10 min. The pellet was washed twice in PBS 1X and the final pellet was resuspended in 100 μl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The sample was then thawed and centrifuged at 3000x g for 5 min. The supernatant was stored at -20°C for further use as template DNA

3.5.1.2b Preparation of template DNA from Salmonella cultures

For preparing bacterial culture lysates as template DNA, a pure colony of *Salmonella* was inoculated into five milliliters of RV broth and incubated at 37°C for 18 h. One point five milliliters of this broth culture was transferred to a Eppendorf tube and centrifuged at 3000 x g for 10 min. The rest of the procedure was as that of 3.5.1.2a

3.5.1.2c Preparation of culture lysates from bacteria other than Salmonella

For testing the specificity of primers, culture lysates were prepared from bacterial cultures maintained in the Department of Microbiology. These included, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*. The procedure was similar to that described in section 3.5.1.2b

3.6. POLYMERASE CHAIN REACTION FOR DETECTION OF GENUS SALMONELLA (*inv*-PCR)

3.6.1 Materials

3.6.1a PCR reaction buffer (10X)

This includes 500 mM KCl, 100 mM Tris-HCl pH 9.0 and 15 mM $MgCl_2$.

3.6.1b Taq DNA polymerase

Taq DNA polymerase enzyme with a concentration of 3 U/μl.

3.6.1c Deoxy ribo nucleotide triphosphate

Deoxy ribo nucleotide triphosphate (dNTP) mix

10mM (2.5 mM of each dGTP, dCTP, dATP and dTTP in equal volume)

All the above reagents were obtained from Bangalore Genei, India Limited.

3.6.1d Primers for genus specific inv- PCR.

Specific primers to detect the *Salmonella* (Genus specific) designed by Oliveira *et al.* (2002) were used. The sequences of the primers were as follows:

*inv*A 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3'-26mer

*inv*A 5'-TCA TCG CAC CGT CAA AGG AAC C -3' –22mer

The primers were custom synthesized by Integrated DNA Technologies, USA.

3.6.2. Method

Polymerase chain reaction was conducted for the detection of *Salmonella* by the method as described by Oliveira *et al.* (2002), using the primer pair *inv*A.

A 25 µl reaction mixture was prepared in 0.2 milliliter thin walled PCR tube (Genei, India). The reaction mixture consisted of the following.

Template DNA	5 µl
Primers	20 picomole of each primer
10 X PCR buffer	2.5 µl
Taq DNA polymerase	1.0 unit
dNTP mix	1 µl
Triple distilled water to	25 µl

The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme.

Initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec and extension at 72°C for 1 min and a final extension at 72°C for 7 min. The whole reaction was carried under the heated lid.

The above-mentioned PCR technique was employed using template DNA prepared from the following materials.

1. Inoculated RV broth ,
2. Bacterial culture lysates.

The product was analysed by submarine agarose gel electrophoresis.

3.6.3 Submarine Agarose Gel Electrophoresis

3.6.3.1 Materials

3.6.3.1a (0.5 M) EDTA (pH 8.0)

Dissolved 18.61 g of EDTA (disodium,dihydrate) in 70 ml of triple distilled water. The pH was adjusted to 8.0 with 1N NaOH. The volume was made upto 100 ml, filtered, autoclaved at 121°C for 15 min at 15 lbs pressure and stored at room temperature.

3.6.3.1b TAE (Tris- acetate EDTA) buffer (50 X) pH 8.0

Tris base	48.40 g
Glacial acetic acid	11.42 ml
0.5 M EDTA pH 8.0	20.00 ml
Distilled water to	1000 ml

Autoclaved at 121°C for 15 min at 15 lbs pressure and stored at room temperature.

3.6.3.1c TAE (1 X)

TAE 50X	2 ml
DW	98 ml

3.6.3.1d Agarose Gel (1.5 per cent)

Agarose low EEO (Genei)	1.5 g
TAE buffer (1X)	100 ml

3.6.3.1e Gel loading buffer (6 X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Sucrose	40.00 g
Distilled water	to 100 ml

Stored at 4°C.

3.6.3.1f Ethidium bromide

Ethidium bromide	100 mg
Distilled water	10 ml

Stored at 4°C in amber coloured bottles

3.6.3.1g DNA molecular size marker

pUC19DNA/*Msp*I digest with fragments 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34 and 26 bp.

The molecular size marker were obtained from Bangalore Genei(India).

3.6.3.2 Method

The PCR product was detected by electrophoresis in 1.5 per cent agarose gel in TAE buffer (1 *X*). Agarose was dissolved in TAE buffer (1 *X*) by heating. When the mixture was cooled to around 50°C, ethidium bromide was added to a final concentration of 0.5 μg/ml. Melted agarose was then poured into clean, dry, gel platform, the edges of which were sealed with adhesive tape and the comb was kept in proper position. Once the gel was set, the comb and adhesive tape were removed gently and the tray containing the gel was placed in the buffer tank. Buffer (TAE 1 *X*) was poured till the gel was completely covered.

Amplified PCR product (5 μl) was mixed with one microliter of 6 *X* gel loading buffer and the samples were loaded in the wells. The pUC19DNA/*Msp*I

digest was used as DNA molecular size marker. Electrophoresis was carried out at 5V/cm for one hour (or) until the bromophenol blue dye migrated more than two-third of the length of the gel.

The gel was visualized under UV transilluminator (Hoefer, USA) and the images were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3.7. POLYMERASE CHAIN REACTION USING SEROVAR SPECIFIC PRIMERS (*Sef*-PCR)

3.7.1 Materials

Except for the primers, the materials used for this technique were the same as those that were used for genus specific-PCR. **(3.6.1)**

3.7.1a Primers for serovar specific - PCR

A primer set designed by Oliveira *et al.* (2002) was used for the molecular typing of *S .pullorum* , *S. gallinarum* and *S . enteritidis*.

The sequences of the two primers were:

*sef*A	5'-GAT ACT GCT GAA CGT AGA AGG -3'	21 mer
*sef*A	5'-GCG TAA ATC AGC ATC TGC AGT AGC - 3'	24 mer

3.7.2 Method

Twenty-five microliter reaction mixture was prepared in 0.2 milliliter PCR tubes with the following proportions.

Template DNA	5.0 µl (boiled culture lysates)
10 X PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl

dNTP mix	2.0 μl
Taq polymerase	1 unit
Primers	20 picomole each *sef*A
Triple distilled water to	25 μl

Template DNA prepared from inoculated RV broth was also used for PCR. The serovar specific-PCR amplification was carried out in an automated thermal cycler (Eppendorf, Master Cycler, Germany) with the following programme.

Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec and extension at 72°C for 1 min and a final extension at 72°C for 7 min. The whole reaction was carried under the heated lid.

Products were analyzed on two per cent agarose gel in TAE 1 X by submarine gel electrophoresis as detailed in 3.6.3.2 Standard molecular weight marker, pUC19DNA/*Msp*I digest was used as DNA molecular size markers to ascertain the size of the DNA fragments.

The specificity of the primers were checked by using DNA prepared from *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli.*

3.8 POLYMERASE CHAIN REACTION FOR DETECTION OF *rfb*S GENE OF AVIAN *SALMONELLA*

3.8.1 Materials

Except for the primers and DNA molecular markers the materials used for this technique were same as that which was used for *inv* PCR. **(3.6.1)**

3.8.1a Primers for rfbS-PCR

Two oligonucleotides based on the sequence of *Salmonella rfbS* gene, Accession No. GAN M29682 were designed by Park *et al.* (2001). These primers were custom synthesized by Integrated DNA Technologies, USA.

The sequences of the two primers were as follows:

SG1 5'-TCA CGA CTT ACA TCC TAC-3' - 18 mer
SG2 5'-CTG CTA TAT CAG CAC AAC -3' - 18 mer

3.8.1b Molecular size marker

pUC18/*Sau3A 1*- pUC18/*Taq 1 Digest*
With fragments 1444,943, 754, 585, 458,341, 258, 153,105,78/75 bp
The molecular size marker was obtained from Bangalore Genei (India).

3.8.2 Method

A 25 µl reaction mixture was prepared in 0.2 milliliter thin walled PCR tube (Genei, India).

Template DNA	5 µl (boiled culture lysate)
Primers	20 picomole of each primer
10 X PCR buffer	2.5 µl
Taq DNA polymerase	1.0 unit
dNTP mix	1 µl
Triple distilled water to	25 µl

The following programme was used - initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The template DNA used were bacterial culture lysate and DNA prepared from RV broth. The product was analyzed by submarine agarose gel electrophoresis as

detailed in 3.6.3.2. Standard molecular weight marker, pUC18/*Sau*3A 1- pUC18/*Taq* 1 Digest was used as DNA molecular size markers to ascertain the size of the DNA fragments.

In order to check the specificity of the primers, DNA prepared from *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* were used in this study.

3.9 RESTRICTION ENZYME ANALYSIS OF *rfb*S GENE PCR PRODUCT

3.9.1. Materials

Two restriction enzymes (RE) viz., *Tfi I* and *Ple I* were used in this study. The enzymes and buffers were obtained from New England bio-labs Pvt. Limited.

PleI (10 units/μl) 5'GAGT↓ C - 3'

(*Dienococcus radiophilus*)

10 XAssay buffer E

TfiI (10 units/μl) 5'G↓AATC-3'

10 X Assay buffer C

Both the above enzymes were chosen based on Park *et al.* (2001)

3.9. 2. Method

The restriction digestion mixture was prepared as follows:

Amplified PCR product	10 μl
10 X RE buffer	2 μl
Restriction enzyme	1 μl
Distilled water	7 μl

Restriction enzyme digestion was performed in Eppendorf Master Cycler, (Germany).

The incubation for *Tfi I* was one hour at 65°C and *Ple 1* was one hour at 37°C.

3.9.3 Electrophoresis of Restricted Product

3.9.3.1 Materials

Except for the 3 per cent agarose and DNA molecular size marker the materials used for this technique were same as that which was used in **3.6.3.1.**

3.9.3.1a Agarose Gel (3 per cent)

Agarose low EEO (Genei)	3.0 g
TAE buffer	100 ml

3.9.3.1b DNA molecular size marker

pBR322DNA/*Alu* I digest with fragments 908, 659,521, 403, 281, 257,226,100/90, 63/57/49 bp.

The molecular size marker were obtained from Bangalore Genei (India).

3.9.3.2 Method

The PCR product was detected by electrophoresis in three per cent agarose gel in TAE buffer (1X). Agarose was dissolved in TAE buffer (1X) by heating. When the mixture was cooled to around 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ml. Agarose was then poured into clean, dry, gel platform, the edges of which were sealed with adhesive tape and the comb was kept in proper position. Once the gel was set, the comb and adhesive tape were removed gently and the tray containing the gel was placed in the buffer tank. Buffer (TAE 1X) was poured till the gel was completely covered.

Restriction digested product (10 µl) was mixed with one microliter of 6 X gel loading buffer and the samples were loaded in the wells. The pBR *322/Alu I* digest was used as DNA molecular size marker. Electrophoresis was carried out at 5V/cm for one hour (or) until the bromophenol blue dye migrated more than two-third of the length of the gel.

The gel was visualized under UV transilluminator (Hoefer, USA) and the images were documented in a gel documentation system (Bio-Rad Laboratories, USA).

3.10 PLASMID PROFILE OF GENUS *SALMONELLA*

3.10.1 Isolation of plasmid DNA

3.10.1.1 Materials

3.10.1.1a Luria Bertani Broth

Yeast Extract	5 g
NaCl	10 g
Tryptone	10 g
Distilled water	to 1 liter

Sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

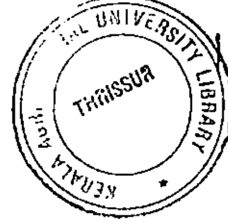
3.10.1.1b Tris HCl (1 M, pH 8.0)

Tris base	12.11 g
Conc. HCl	0.2 ml

The above ingredients were dissolved in 90 ml of triple distilled water. The volume was made upto 100 ml with distilled water and sterilized by autoclaving.

3.10.1.1c Sodium acetate (3 M, pH 4.8)

Sodium acetate	40.81 g



72425

Distilled water to 100 ml

pH was adjusted to 4.8 with glacial acetic acid and stored at 4°C

3.10.1.1d NaOH (1N)

NaOH	4 g
Distilled water to	100 ml

Stored at room temperature.

3.10.1.1e Tris-EDTA Glucose (TEG) buffer pH (8.0)

Tris (0.25M)	2.5 ml of 1M Tris
Glucose (50 mM)	9.008 g
EDTA (10 mM)	2.0 ml of 0.5 M EDTA

Distilled water to 100 ml

Autoclaved at 121°C for 15 min at 15 lbs pressure and stored at room temperature.

3.10.1.1f SDS-NaOH solution

10 per cent SDS	0.5 ml
1N NaOH	1.0 ml
Triple distilled water	3.5 ml

The solution was prepared fresh each time.

3.10.1.1g Phenol: chloroform: isoamyl alchocol

phenol	25 ml
chloroform	24 ml
iso amyl alchocol	1 ml

3.10.1.1h Chloroform: isoamyl alchocol

chloroform	24 ml
isoamyl alchocol	1 ml

3.10.1.1i Ethanol

3.10.1.1 j Tris –EDTA buffer (TE 1 X) pH 7.8

Tris		1ml(stock solution)
0.5 M EDTA		0.2 ml
Triple distilled water	to	100 ml

3.10.1.1k Tris-borate EDTA buffer (TBE 5 X) pH 8.0

Tris base		54 g
Boric acid		27.5 g
0.5 M EDTA		2 ml
Triple distilled water	to	1000 ml

3.10.1.1l TBE 1 X

TBE 5 X	20ml
Triple distilled water to	100 ml

3.10.1.2 Method

One milliliter of *Salmonella* culture was inoculated in to 10 ml of Luria Bertani broth and incubated at 37°C for eight hours.

The broth was centrifuged at 8000 x g for 15 min. Resuspended the pellet in 100 µl of TEG buffer containing lysozyme at a concentration of 10 mg/ ml and was kept on ice for 15 min. To this mixture, 200 µl SDS-NaOH was added and gently mixed until the solution became translucent. The tube was further

incubated on ice for 15 min. To this was added 150 μl of 3 M sodium acetate and incubated on ice for another 15 min. The mixture was centrifuged at 12000 x g for 30 min at 4°C.

The supernatant was carefully transferred into a fresh Eppendorf tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by gentle inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred to a fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently by gentle inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred into a fresh Eppendorf tube, added double the volume of ice-cold ethanol and allowed the plasmid DNA to precipitate at -70°C overnight. The tube was then thawed and centrifuged at 10,000 x g for 15 min. The DNA pellet was washed in 70 per cent ethanol and the final pellet was resuspended in 20 μl of TE buffer. Plasmid DNA from *Escherichia coli* V517 procured from the Microbial Type Culture Collection (MTCC), Chandigarh, was prepared in a similar manner.

3.10.2 Submarine Agarose Gel Electrophoresis

The isolated plasmid DNA was analysed by submarine agarose gel electrophoresis as detailed in 3.6.3.2, except that 0.8 per cent agarose gel in 1 X TBE buffer was used.

Approximately 20 μl of plasmid DNA was mixed with one microliter of 6X gel loading dye (one μl) and loaded into the wells. *Escherichia coli* V517 plasmid DNA was used to ascertain the size of the plasmids. Electrophoresis was carried out at 40V till the dye reached near the edge of the gel.

The DNA fragments were viewed under UV transilluminator and photographed using a gel documentation system (Bio-Rad, USA).

3.11 MULTIPLEX PCR FOR VIRULENCE PLASMID

3.11.1 Materials

Except for the spvC primers, the materials used for this technique were the same as those that were used for genus specific- PCR. **(3.6.1)**

3.11.1a. Primers for virulence plasmid

spvC 1 5' ACT CCT TGC ACA ACC AAA TGC GGA-3'-24 mer
spvC 2 5' TGT CTC TGC ATT TCG CCA CCA TCA-3'-24 mer

3.11.2. Method

A 25 µl reaction mixture was prepared in 0.2 milliliter PCR tubes with the following proportions

Template DNA	5.0 µl (boiled culture lysates)
10 X PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
dNTP mix	2.0 µl
Taq DNA polymerase	1 unit
Primers	20 picomole each *inv*A primers, *svpC*1 and *svpC*2
Triple distilled water to	25 µl

The virulence plasmid-PCR amplification was carried out in an automated thermal cycler (Eppendorf, Master Cycler, Germany) with the following programme.

Initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 2 min and a final extension at 72°C for 10 min. The whole reaction was carried under the heated lid.

Products were analyzed on two per cent agarose gels in TAE 1 X by submarine gel electrophoresis as detailed 3.6.3.2. Standard molecular weight marker pUC19DNA/*Msp*I digest was used as DNA molecular size markers to ascertain the size of the DNA fragments.

By using DNA, prepared from *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli,* the speficity of the primers were checked.

3.12 ALLELE SPECIFIC PCR FOR *SALMONELLA GALLINARUM*

3.12.1 Materials

Except for the primers, the materials used for this technique were the same as those that were used for genus specific-PCR. **(3.6.1)**

3.12.1.1 Primers for Allele specific PCR

*rfb*SF 5'-GTA TGG TTA TTA GAC GTT GTT-3'- 21 mer
(Forward primer for both *Salmonella gallinarum, Salmonella pullorum*)

*rfb*SG 5'-TAT TCA CGA ATT GAT ATA CTC-3'- 21 mer
(Specific for *Salmonella gallinarum*)

*rfb*SP 5'-TAT TCA CGA ATT GAT ATA TCC-3'- 21 mer
(Specific for *Salmonella pullorum*)

3.12.2 Method

A 25 µl reaction mixture was prepared in 0.2 milliliter PCR tubes with the following proportions.

3.12.2.1 Reaction mixture for Salmonella gallinarum

Template DNA	5.0 µl (boiled culture lysates)
10 X PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
dNTP mix	2.0 µl

Taq DNA polymerase	1 unit
Primers	25 picomole each *rfb*SF, *rfb*SG
Triple distilled water to	25 µl

3.12.2a Reaction mixture for Salmonella pullorum

Template DNA	5.0 µl (boiled culture lysates)
10X PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
dNTP mix	2.0 µl
Taq polymerase	1 unit
Primers	25 picomole each *rfb*SF, *rfb*SP
Triple distilled water to	25 µl

PCR assays were performed by denaturation at 94 ° C for 5 min, followed by 30 three-step cycles including denaturation at 94 °C for 1 min, annealing at 60 ° C for 1 min, extension at 72°C for 1min followed by final extension at 72°C for 5 min.

Products were analyzed on two per cent agarose gels in TAE 1 X by submarine gel electrophoresis as detailed (3.6.3.2). Standard molecular weight marker pUC19DNA/*Msp*I digest was used as DNA molecular size markers to ascertain the size of the DNA fragments.

In order to check the speficity of the primers, DNA prepared from *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* was used in this study.

3.13 GENE SEQUENCING OF *rfbS* PCR AMPLICON

3.13.1 Purification of PCR Product

3.13.1.1 Electrophoresis

The PCR products (*rfbS*gene) were electrophoresed on 1.5 per cent agarose gel as described for the detection of PCR products. The gel was transferred to the UV transillurninator and the band was visualized under preparative UV source. Using a sharp scalpel blade, the agarose containing the band of interest was cut with minimum amount of free agarose.

3.13.1.2 Electroelution

A piece of dialysis tubing about seven centimeters long was taken and one end was sealed. The slice of gel containing the band of interest was pushed into the dialysis tube. About 500 µl of TBE buffer was added to the dialysis tubing so that the gel slice was completely immersed without any bubbles and the other end of the dialysis tubing was sealed. The dialysis tubing containing the agarose slice was immersed in a shallow layer of 1 X TBE buffer in an electrophoresis tank. Electroelution was carried out at 4-5V/cms for three hours. During this time the DNA was electroeluted out of the gel into the inner wall of the dialysis tube. The polarity of the current was reversed for one minute to release the DNA from the wall of the dialysis tube. At the end of electroelution, the bag was opened and the entire buffer surrounding the gel slice was carefully transferred to a sterile Eppendorf tube.

3.13.1.3 DNA cleaning and precipitation

To Eppendorf tube containing the buffer was added an equal volume of chloroform: isoamyl alchohol mixture (24:1) and spun at 12,500 x g for three minutes at 4°C. The top aqueous phase containing the DNA was carefully transferred to a sterile Eppendorf tube without disturbing interphase. To this DNA in the Eppendorf tube 1/10 volume of 3 M sodium acetate (pH 4.8) was

added, followed by the addition of two volumes of ice cold ethanol and kept at -20°C for 15 min. The mixture was then centrifuged at 12,500 x g for 15 min at 4°C. The supernatant was decanted and 500 µl of 70 per cent ethanol (ice cold) was added to the pellet. The tube was spun at 12,500 x g for 2 min. Removed the supernatant and the DNA pellet was dissolved in sterile triple glass distilled water. The purified PCR product was then directly used for sequencing.

3.13.2 Sequencing of PCR Product

The purified PCR product was directly sequenced by Sanger's dideoxy chain termination method using ABI PRISM Model 310 version 3.4.1. Primers *rfb*S1 and *rfb*S2 were used as sequencing primers. Sequencing was carried out at the School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu.

Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) network provided by the National Centre for Biotechnology Information (NCBI).

Results

4. RESULTS

4.1 ISOLATION OF *SALMONELLA*

Isolation of *Salmonella* was attempted from clinical samples collected from 155 birds, including 119 chicken, 5 ducks, 19 quails and 12 turkeys. Thirty-four environmental samples were also screened for *Salmonella*.

Fifty-one ailing/dead birds were subjected to detailed post mortem examination. The gross lesions in the birds comprised of swollen friable liver that was dark red or almost black, with a distinctive coppery bronze sheen, enlarged spleen and oophoritis. (Fig. 1 and 2).

Bio-materials like cloacal swabs collected from the birds and environmental samples like hatcher swab, droppings, drag swab, feed, water, setter tray swab and egg surface swab were initially resuscitated in BPW pre-enrichment broth followed by enrichment in RV broth. In enrichment broth growth was indicated by change of colour of broth from green to straw yellow. They were subcultured on to selective media. From the dead/ailing birds, samples like liver, spleen, ovary, caecal tonsil and intestine were directly plated on to MCA and BGA.

On MCA, colonies that appeared transparent and colourless were regarded as suspect of *Salmonella* (Fig 3). On BGA plates, pale pink colonies of size one to two-millimeter diameter were regarded as suspect of *Salmonella* spp. They were usually surrounded by a pink zone in the agar.

Although bacterial isolation was attempted from samples collected from 155 birds, samples from only 15 birds yielded bacterial colonies suggestive of *Salmonella* on MCA and BGA, even after 48 h of incubation at 37°C. From these 15 birds multiple isolations were made from sources like cloacal swabs, liver and spleen. One ovary sample and two intestinal samples of the above 15

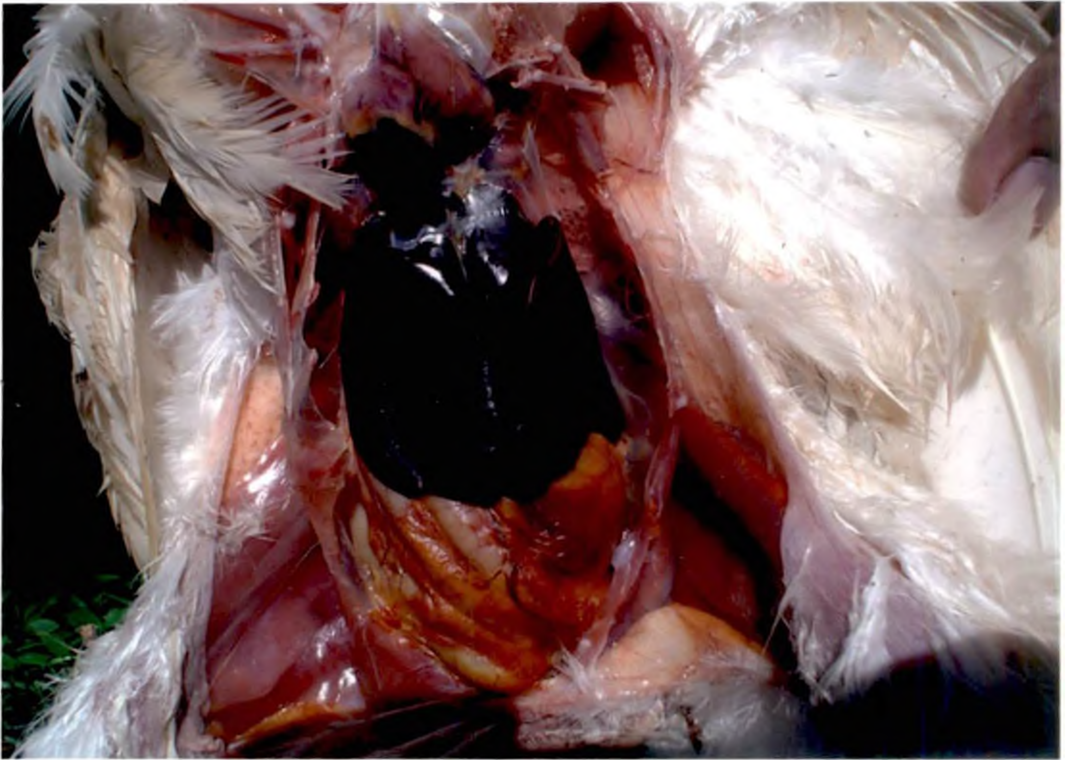


Fig. 1. Coppery bronze liver

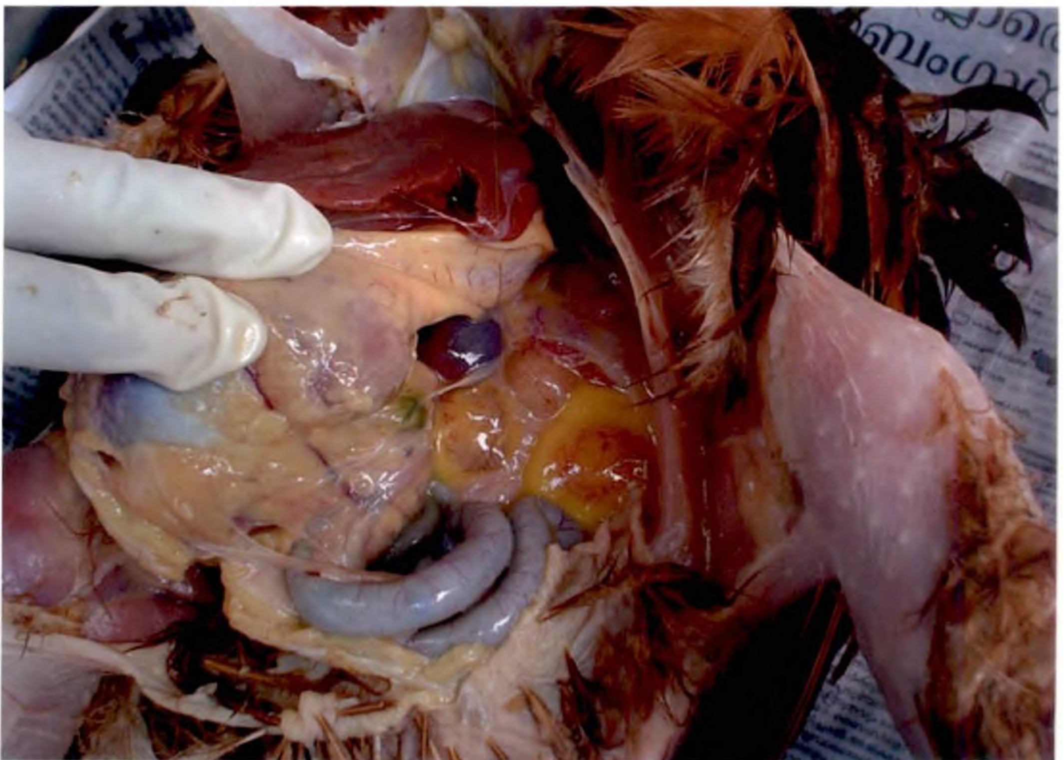


Fig. 2. Oophoritis

birds were also positive for *Salmonella*. The individual colonies which were suspected for *Salmonella* species were subcultured by pure culture techniques and they were maintained on nutrient agar slant for further characterization (Table 4).

Table 4. Isolation of *Salmonella* from poultry samples

Source	Nature of material					Name of the isolate
	Cloacal swab	Liver	Spleen	Ovary	Intestinal content	
Bird No. 22	+	+	+	-	-	PS 1
Bird No. 25	+	+	+	+	-	PS 2
Bird No. 27	+	+	+	-	+	PS 3
Bird No. 29	+	+	+	-	-	QS 1
Bird No. 41	+	+	+	-	-	PS 4
Bird No. 56	+	+	+	-	+	PS 5
Bird No. 81	+	+	+	-	-	QS 2
Bird No. 93	+	+	+	-	-	PS 6
Bird No. 105	+	+	+	-	-	PS 7
Bird No. 107	+	+	+	-	-	PS 8
Bird No. 111	+	+	+	-	-	PS 9
Bird No.119	+	+	+	-	-	PS 10
Bird No. 123	+	+	+	-	-	PS 11
Bird No. 138	+	+	+	-	-	PS 12
Bird No. 142	+	+	+	-	-	PS 13

None of the environmental samples presented colonies on MCA and BGA, which were suggestive of *Salmonella*.

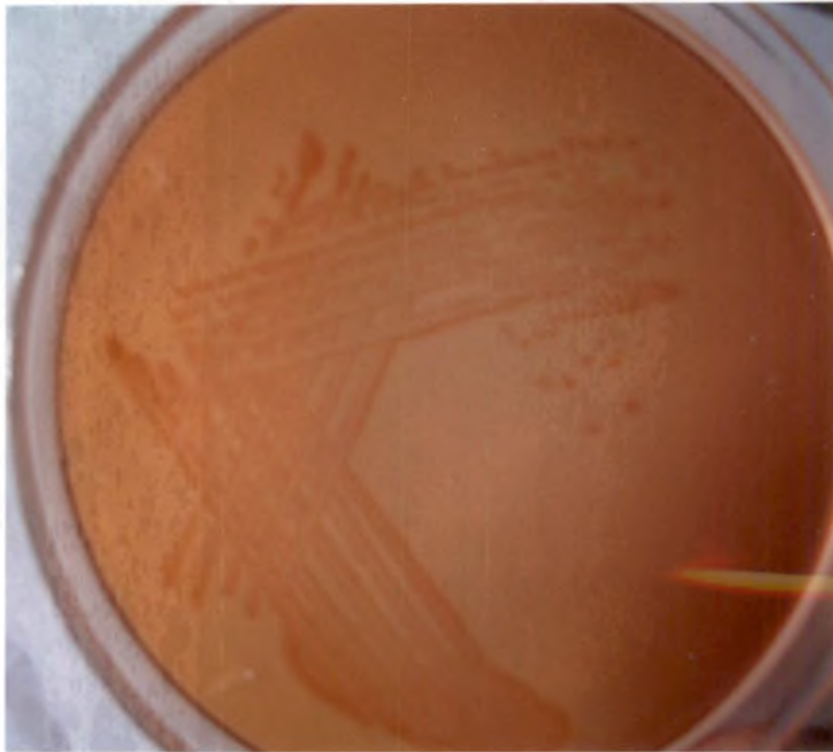


Fig. 3. Colonies on Mac Conkey's agar

Fig. 4. TSI agar
Tube 1 - Control
Tube 2 & 3 - Acid butt,alkaline slant with H2S

4.2 IDENTIFICATION OF THE ORGANISM

4.2.1 First Stage

Altogether 13 bacterial isolates suggestive of *Salmonella* were obtained from chicken (named PS1 – PS13) and two isolates from quails (QS1 and QS2). All isolates were Gram-negative bacilli. They were non motile and grew aerobically. All were catalase positive, oxidase negative and fermented glucose.

4.2.2 Second Stage

In the second stage biochemical tests, all the isolates tested were indole negative, methyl red positive and Voges-Proskauer negative, urease negative, phenyl alanine deaminase negative, reduced nitrate, ornithine decarboxylase positive and citrate utilization negative. In TSI agar, acid butt (yellow), alkaline slant (pink) with H_2S (black) were obtained for all the isolates. (Fig. 4).

With regard to the fermentation of the sugars all the isolates fermented dulcitol, maltose, arabinose, trehalose, and mannitol. They could not utilize sucrose, cellobiose and lactose. Xylose could be fermented only by isolates PS 1, 2, 3, 5, 6, 13, QS1 and QS2. (Table 5). Sorbitol was not utilized by two isolates one each from chicken and quail.

4.3 ANTIBIOGRAM

Antibiogram of *Salmonella* isolates indicating the susceptibility and resistance to various antibiotics/antibacterial agents is presented in Table 6.

All isolates were sensitive to enrofloxacin, pefloxacin ,chloramphenicol and ciprofloxacin.

Table 5. Second stage biochemical tests of isolates

Tests	PS1	PS2	PS3	PS4	PS5	PS6	PS7	PS8	PS9	PS10	PS11	PS12	PS13	QS1	QS2
Indole Production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl Red Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenyl alanine deaminase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine Decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sugar Fermentation															
Xylose	+	+	+	-	+	+	-	-	-	-	-	-	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+

Table 6. Antibiogram of *Salmonella* Isolates

	PS 1	PS 2	PS 3	PS 4	PS 5	PS 6	PS 7	PS 8	PS 9	PS 10	PS 11	PS 12	PS 13	QS 1	QS 2
Ampicillin	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S
Cloxacillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amoxycillin	R	S	S	S	S	R	R	S	S	S	S	S	S	S	S
Enrofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Pefloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	R	S	S	S	R	S	R	R	S	R	S	R	S
Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Co-trimoxazole	S	S	S	R	S	S	S	S	S	S	R	S	S	S	S
Furazolidone	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Twelve isolates representing 80.00 per cent of the isolates tested were found to be sensitive to ampicillin and amoxycillin , 86.66 per cent isolates tested were found to be sensitive to co-trimoxazole.

Nine isolates representing 60.00 per cent of the isolates tested were found to be sensitive to gentamicin.

All the isolates were resistant to cloxacillin, tetracycline and furazolidone.

4.4 SEROTYPING OF THE ISOLATES

Six isolates which were sent to National Salmonella and Escherichia Center, Kasauli, for serotyping. Two isolates (PS 5 and PS 6) were serotyped as *Salmonella gallinarum.*

4.5 POLYMERASE CHAIN REACTION FOR DETECTION OF GENUS *SALMONELLA*

The expected 284 bp amplified product specific for genus *Salmonella* were obtained when DNA extracted from 46 RV broth enriched samples were subjected to PCR. DNA from all the 15 isolates also presented 284 bp products. Analysis of the electrophoresed gel under UV transilluminator revealed the presence of a 284 bp band in RV broth enriched samples and the representation of isolates PS1, PS2, PS3, PS4 and QS1 is shown in fig.5. In the negative control no amplification product was detected. The results are given in Table 7.

Table 7. Results of materials used for Rappaport-Vassiliadis - Polymerase Chain Reaction (RV-PCR) assay

Sl. No.	Nature of material	No. of samples Examined	Positive by genus specific *inv* PCR	Positive by serovar specific *sef* PCR	Positive by *rfb*S gene specific PCR
1	Cloacal swab	155	42	42	13
2	Intestine	25	2	2	2
3	Hatcher tray swab	10	1	1	0
4	Droppings	4	0	0	0
5	Drag swab	4	1	1	0
6	Water	4	0	0	0
7	Setter tray swab	4	0	0	0
8	Egg surface swab	4	0	0	0

No amplification product was detected when *inv* primers were used to amplify the DNA prepared from *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*.

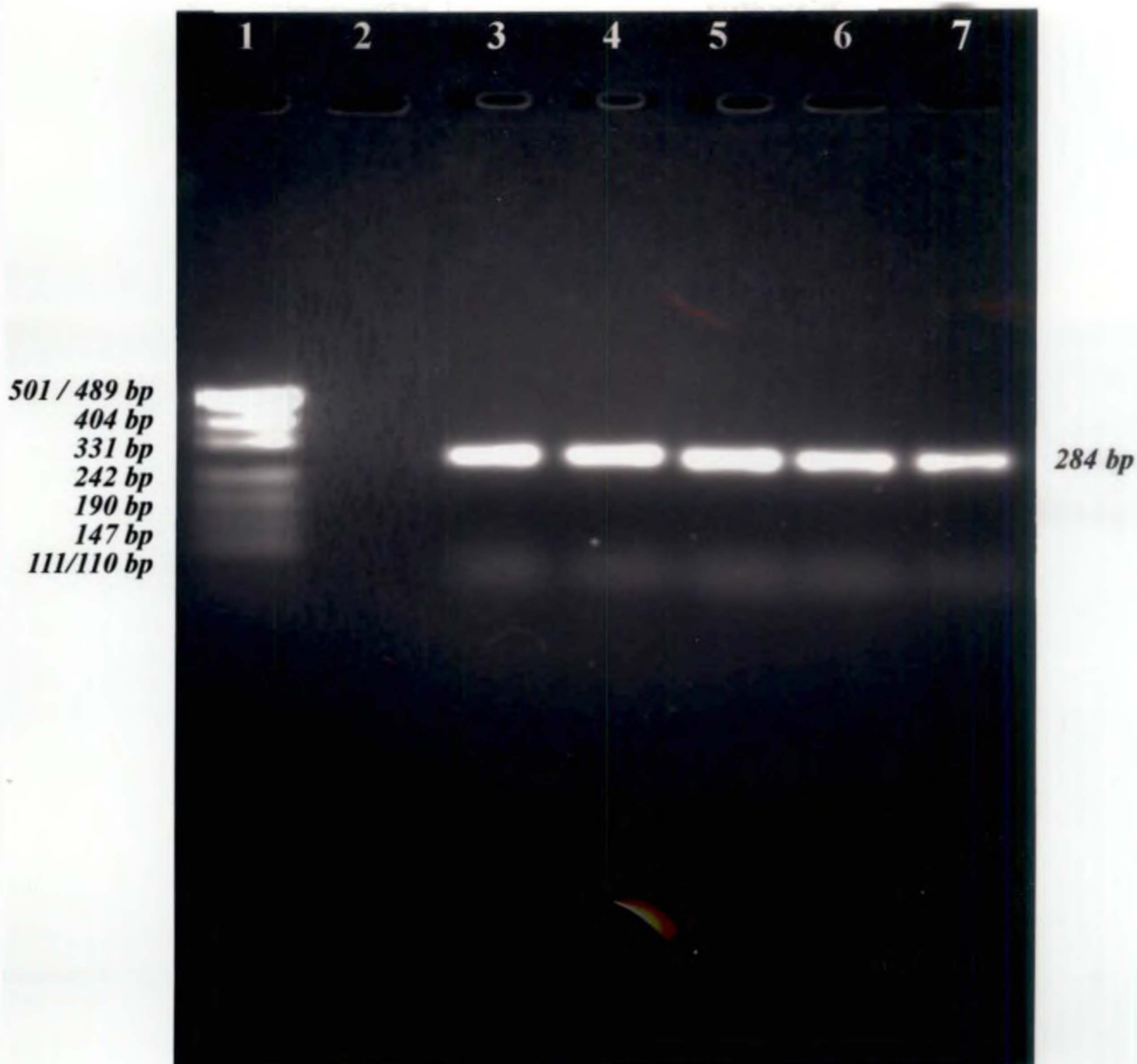


Fig. 5. ***Salmonella* genus specific *inv* PCR**

Lane 1 pUC 19 DNA / *Msp* I digest marker

Lane 2 Negative control

Lane 3 - 6 PS 1, PS 2, PS 3, PS 4

Lane 7 QS 1

4.6 POLYMERASE CHAIN REACTION USING SEROVAR SPECIFIC PRIMERS

A primer set designed by Oliveira *et al.* (2002) was used for the molecular typing of *S. pullorum* , *S. gallinarum* and *S . enteritidis*.

DNA extracted from inoculated RV, all the 13 isolates of *Salmonella* from chicken as well as the quail isolates QS1 and QS2, which were found to be positive by *inv*-PCR, were further subjected to *sef*-PCR, using the primer pairs *sefA* . Analysis of the electrophoresed gel under UV transilluminator revealed the presence of a 488 bp band in template DNA prepared from RV enriched broth samples and all the 15 isolates. Fig.6. Depicts the electrophoretogram of isolates PS1, PS2, PS3, PS4 and QS1.

In the negative control no amplification product was detected.

Primer pairs *sefA* did not amplify the DNA prepared from unrelated bacterial species such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*.

4.7 POLYMERASE CHAIN REACTION FOR DETECTION OF *rfb*S GENE OF AVIAN *SALMONELLA*

Two oligonucleotides based on the sequence of *Salmonella rfbS* gene, Accession No. GAN M29682 were designed by Park *et al.* (2003) was used in this study.

DNA extracted from inoculated RV broth, all the 13 fowl isolates of *Salmonella* as well as the quail isolates QS1 and QS2, which were found to be positive by both *inv* and *sefA*-PCR, were subjected to *rfb*S gene PCR, using the primer pairsSG1 and SG2. Agarose gel electrophoresis of the amplified PCR product was carried out along with a negative control and a molecular size marker (pUC18/*Sau3A* 1- pUC18/*Taq* 1 Digest) in 1 X TAE buffer.

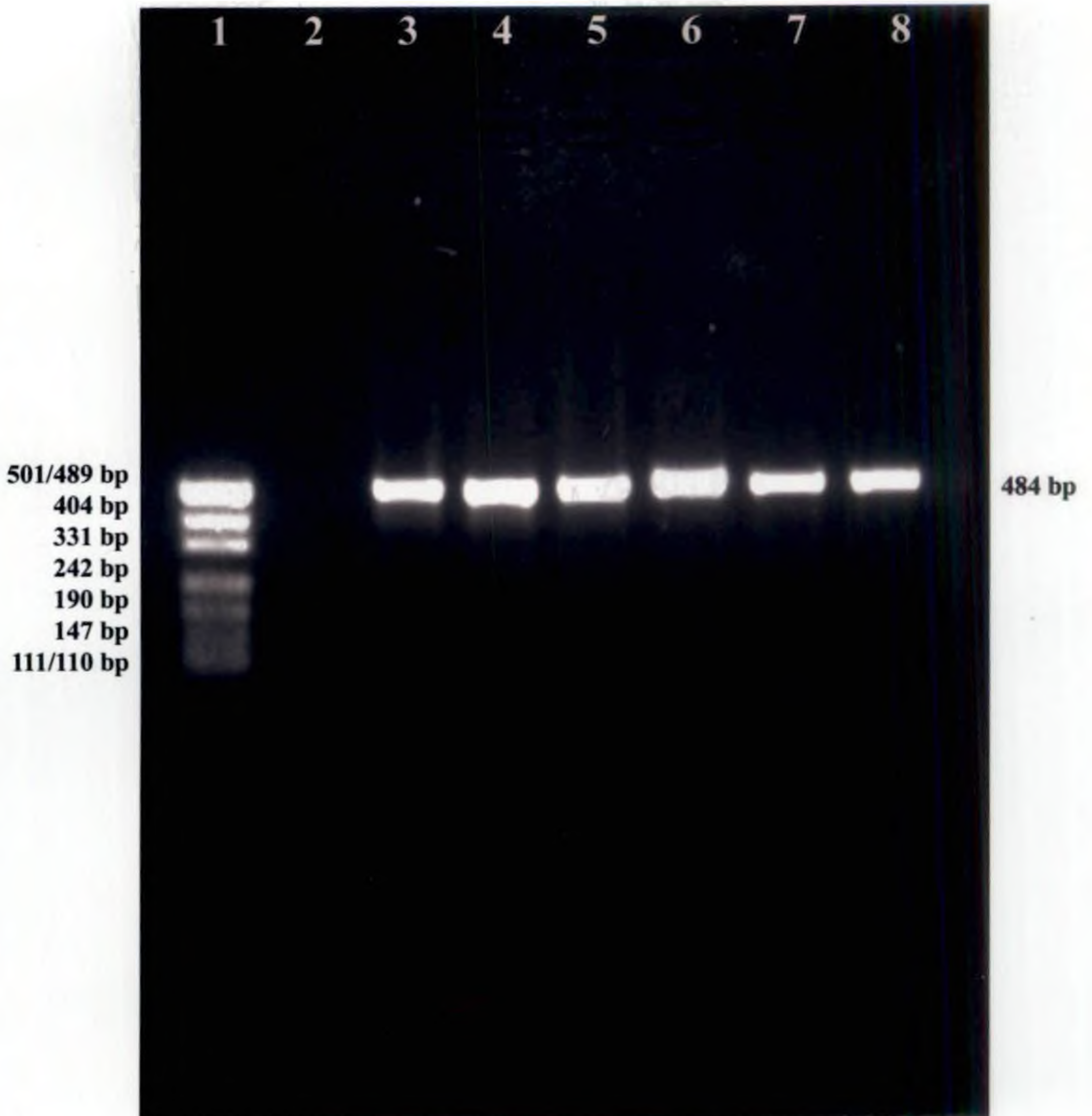


Fig. 6. Serovar specific *sef* PCR

Lane 1 pUC 19 DNA/*Msp* I digest marker

Lane 2 Negative control

Lane 3 -7 PS 1, PS 2, PS 3, PS 4, PS 5

Lane 8 QS 1

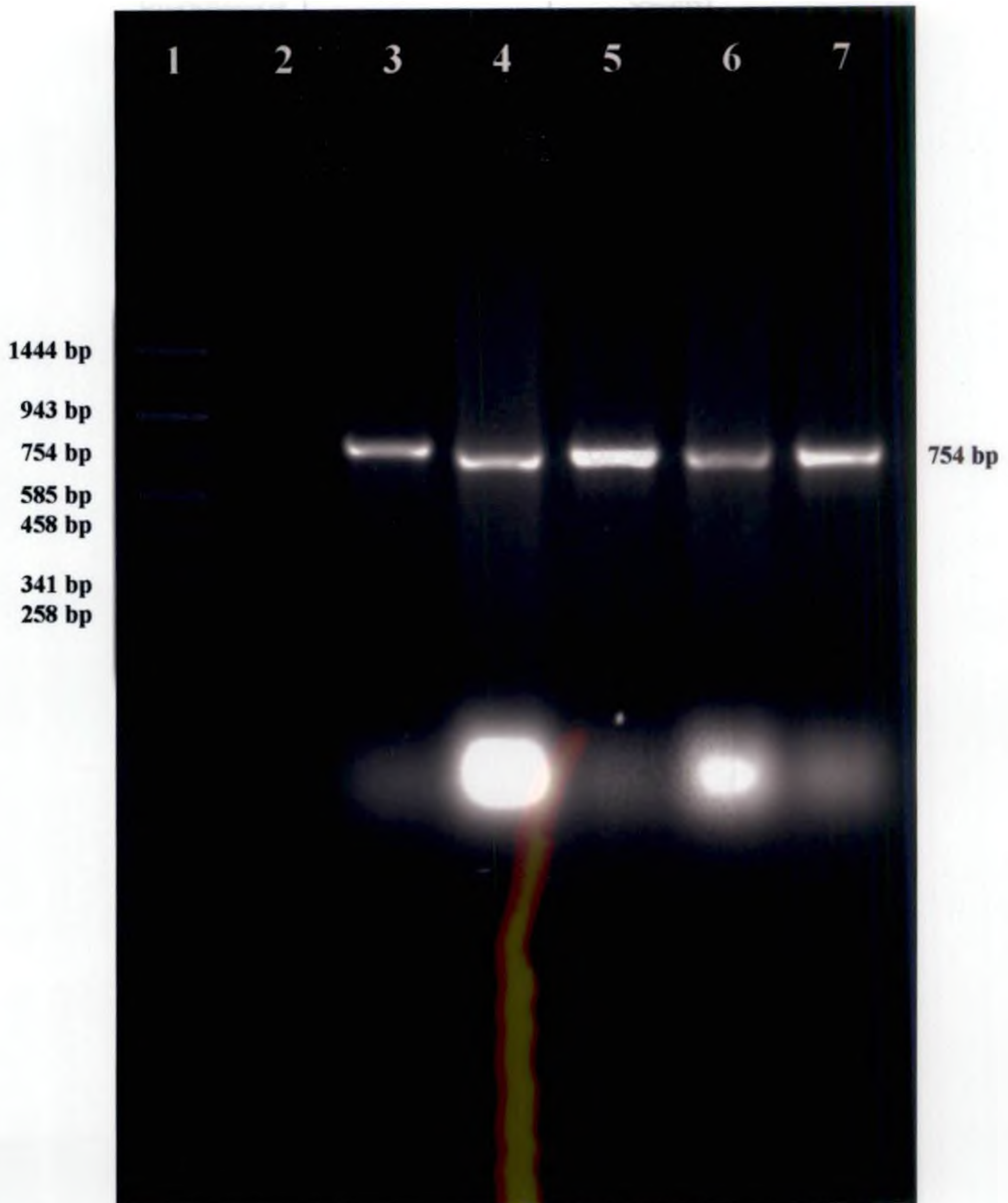


Fig. 7. ***rfbS*** **gene specific PCR**

Lane 1 pUC 18 /*Sau* 3A I - pUC 18 / *Taq* I Digest

Lane 2 Negative control

Lane 3 - 6 PS 1, PS 2, PS 3, PS 4

Lane 7 QS 1

Analysis of the electrophoresed gel under UV transilluminator revealed the presence of a 754 bp band in 15 out of 46 RV broth enriched samples. DNA from all the 15 isolates also presented 754 bp products. Fig.7. Depicts the electrophoretogram of isolates PS1, PS2, PS3, PS4 and QS1.

In the negative control no amplification product was detected.

DNA prepared from unrelated bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* did not reveal any amplification.

4.8 RESTRICTION ENZYME ANALYSIS OF *rfb*S GENE PCR PRODUCT

Two restriction enzymes viz., *Tfi I* and *Ple I* were used in this study.

Restriction digestion analysis of amplicon DNA of all isolates with *Tfi I*, specific for *Salmonella gallinarum* yielded the expected 235 bp product. When isolates were subjected to restriction digestion with *Ple I* specific for *Salmonella pullorum*, there was no digestion of DNA. From the restriction enzyme analysis it was concluded that all the 15 isolates were *Salmonella gallinarum*. The representation of isolates PS1, PS2, PS3, PS4 and QS1 is shown in fig.8.

4.9 PLASMID PROFILE OF *SALMONELLA GALLINARUM* ISOLATES

All the 15 isolates harbored plasmids. The number of plasmids varied from two to four. Isolate PS1 which harbored four plasmids (48.06 kbp, 5.12 kbp and two low molecular weight bands above 3.36 kbp) was categorized in profile I while the rest of the isolates, each of which carried a single high molecular weight plasmid (48.06 kbp) and a low molecular weight plasmid (above 3.36 kbp) were grouped in profile II. (Fig. 9).

Fig. 8. PCR – RFLP of *rfb*S PCR product digested with *Tfi* I

Lane 1 pBR 322 DNA/*Alu*I digest marker

Lane 2 Undigested 754 bp PCR product

Lane 3 - 6 PS 1, PS 2, PS 3, PS 4

Lane 7 QS 1

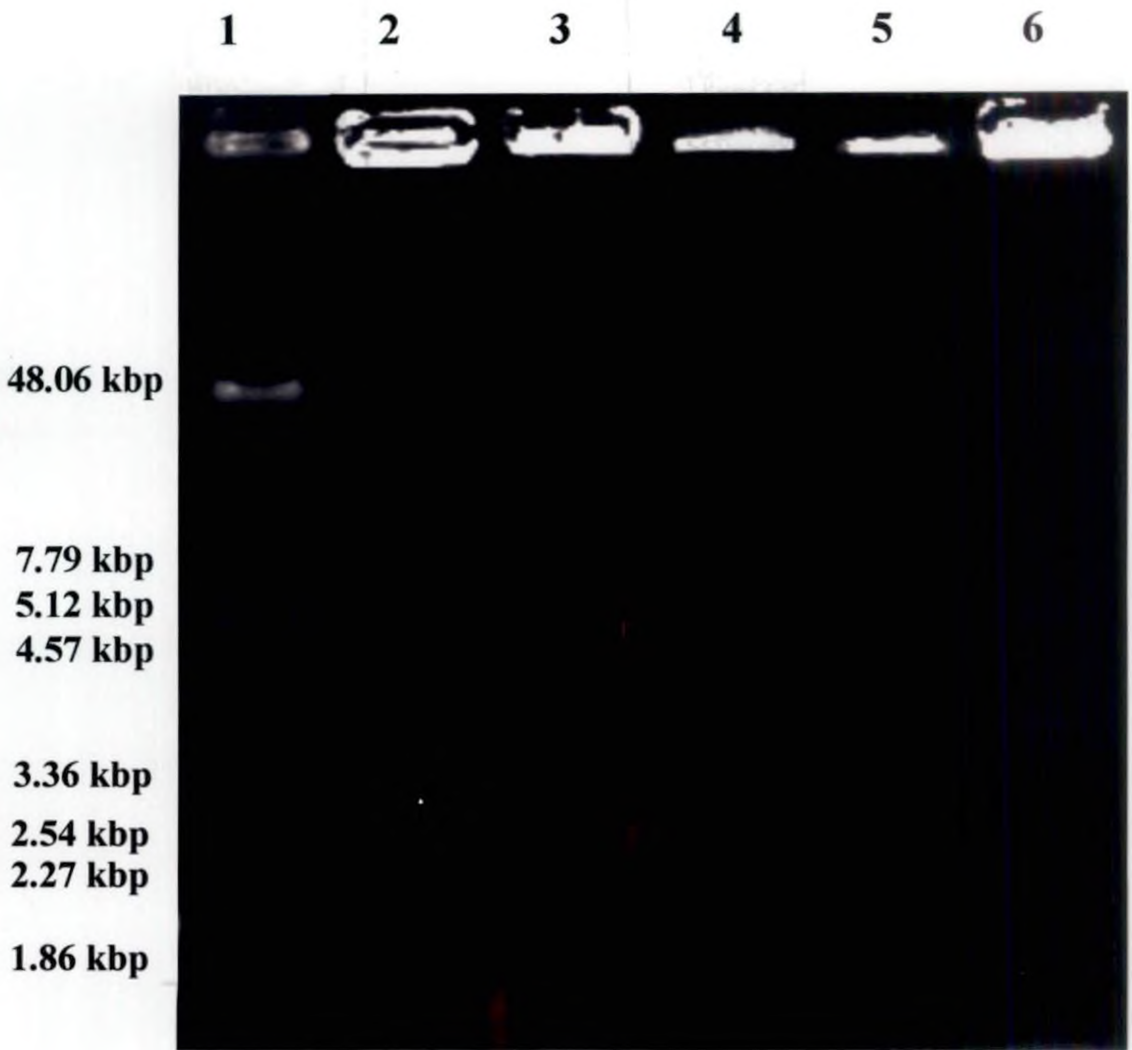


Fig. 9. Plasmid profiles of *Salmonella gallinarum*

Lane 1 *E.coli* V517

Lane 2 – 5 profile II(PS 2,PS 3,PS 4,QS 1)

Lane 6 profile I (PS 1)

4.10 MULTIPLEX PCR FOR VIRULENCE PLASMID

Two pairs of oligonucleotide primers were prepared according to the sequences of the chromosomal *invA* and plasmid *spvC* genes were used in this study. The expected 284 bp amplicon (from the *invA* gene) and 571 bp amplicon (from the *spvC* genes) were obtained in all the 15 isolates. The above results showed that all the isolates were harboring virulence plasmids. Fig.10. Depicts the electrophoretogram of isolates PS1, PS2, PS3, and PS4 and QS1.

Primer pairs *SpvC* 1 and *SpvC* 2 did not amplify the DNA prepared from unrelated bacterial species such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*.

4.11 ALLELE SPECIFIC PCR FOR *SALMONELLA GALLINARUM*

The primers for allele-specific PCR amplification of *rfbS* gene were selected based on sequences described by Park *et al.* (2001)

The expected 187 bp amplicons specific for *Salmonella gallinarum* by using primer pairs (*rfbSF, rfbSG*) were obtained in all the 15 isolates. None of the template DNA amplified when *Salmonella pullorum* specific primers (*rfbSF, rfbSP*) were used. Fig.11. Depicts the electrophoretogram of isolates PS1, PS2, PS3, PS4 and QS1.

There was no amplification when the primers were used to amplify the DNA prepared from *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*.

4.12 GENE SEQUENCING OF *rfb*S PCR AMPLICON

The *rfb*S PCR product with an approximate molecular size of 754 bp was sequenced by Sanger's dideoxy chain termination method.

The amplified product was electroeluted from the gel and used for sequencing. The eluted product was further checked by electrophoresis for the

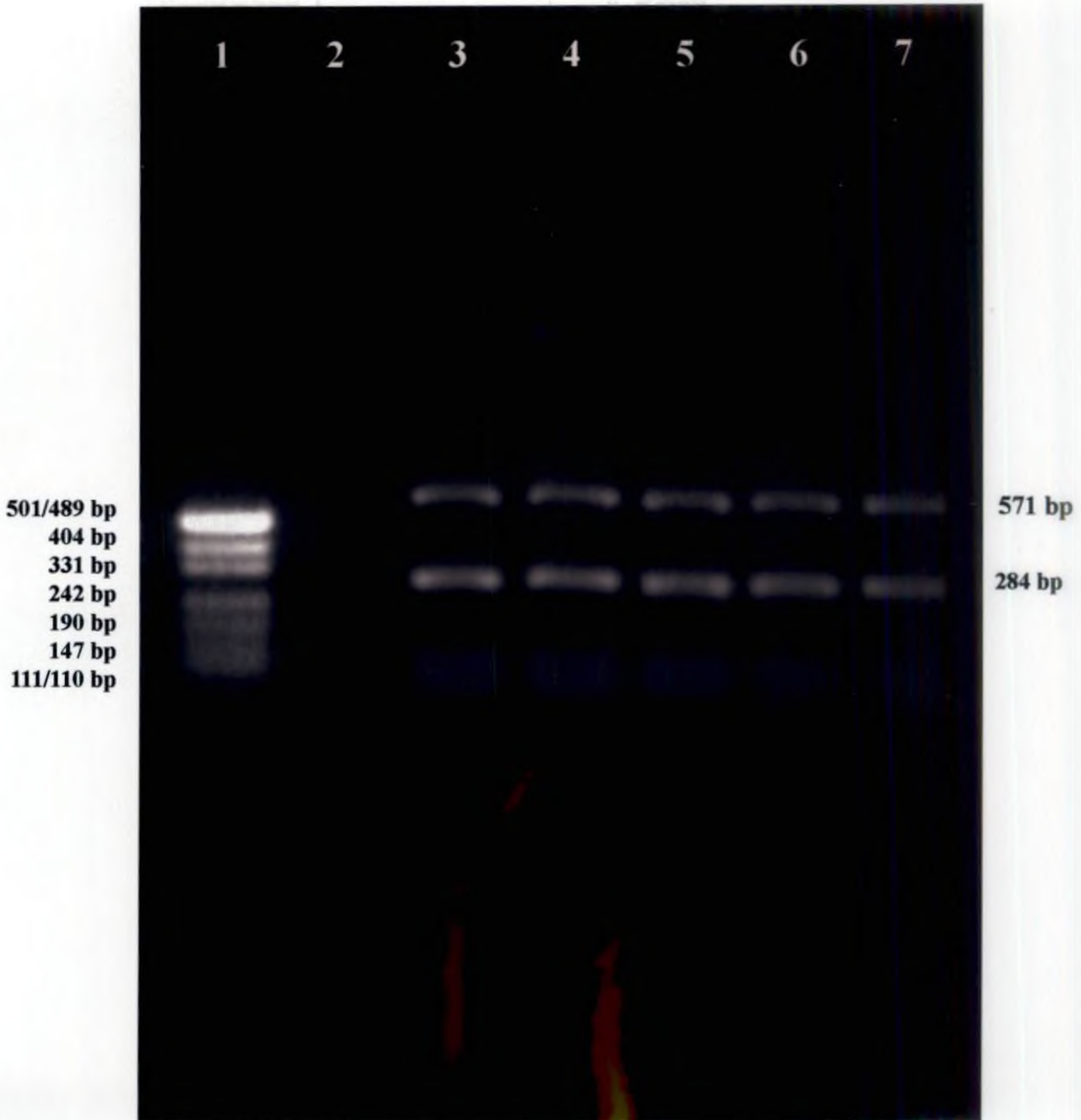


Fig. 10. Multiplex PCR for virulence plasmid

Lane 1 pUC 19 DNA/*Msp*I digest marker

Lane 2 Negative control

Lane 3 - 6 PS 1, PS 2, PS 3, PS 4

Lane 7 QS 1

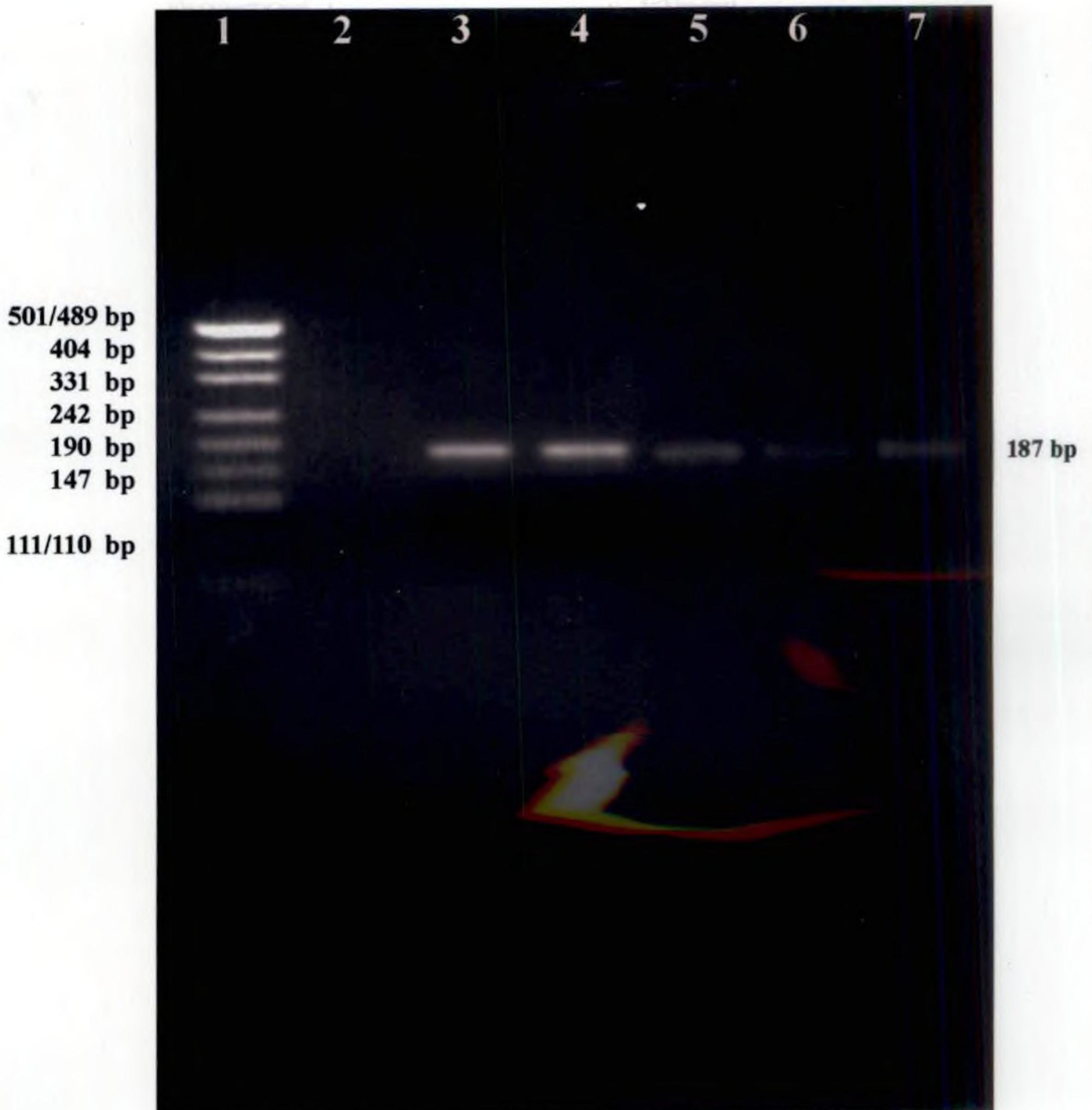


Fig. 11. ***Salmonella gallinarum*** **allele specific PCR**

Lane 1 pUC 19 DNA/*Msp*I digest marker

Lane 2 Negative control

Lane 3 - 6 PS 1, PS 2, PS 3, PS 4

Lane 7 QS 1

presence of any non-specific bands or oligonucleotide primers. Sequencing revealed the presence of 656 base pairs and is shown in figure 12. The sequence has been submitted to the GenBank and has been assigned the accession No AF 442573 ATCC 9184

Figure 12. *rfbS* gene Sequence of SG2

TCCATCTTGNNTTCTTTGCCCCTCAATTCATTATAGAATTGCTTTTTGT
GATATTTTTAACAGTCTCTACATATTCACGAATTGATATAGTCTTTCCA
CTACCAACTTCAATACTATGAAATTTGGGGAAATTATTAACATTATTT
ATAATACAATCGAACGCTGTTAGTAGATCTTTTATATACAAGAAATCT
CTCTGTTGCAAACCAGATGTTAACTTTACTGGCTGGTTACTTAAACAA
CGTCTAATAACCATACTGGTAAACTTATCGTCTCCATCAAAAGCTCCA
TAGAAATGCTCCAATTTTAACTCTATATATTTACCACAAACTTTATCTA
TAATGGCGGCGGCGAGTTCATTTGCTTTTTGTTTAGTATATGCATATA
AACTCGTATTTGGTGGCAGTGATGTTCCACAATTTATGAATACTGCAT
CAAGTGATGAGATAGATTCTAATACTCTGATAGGCATAAGAATATTGC
TTTCTATTAAAGCTGTTGCAGGTTCGTTATATCTTCCATAGCAAGCAAT
AGTGTTAATAATAATATTCGGTTCAAATTCTACTATTTTTTCGATCCAA
TTATTTTCTGTCGTGTAAATAATATTATTTATGGGTAGCTTCATTGTTC
CTCTTCCTTGCTAAGCCAATCCAGTATGTCGACTTTC-656 bp

Sequence similarity searches were performed with BLAST provided by the NCBI. The sequence had 99 per cent identity with *Salmonella gallinarum* (Accession No AF 442573 ATCC 9184).

The result of the alignment is shown in figure 13.

Figure 13. BLAST Results

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.10 [Oct-19-2004]

Sequence 1 lcl|seq_1 **Length** 721 (1 .. 721)

Sequence 2 lcl|seq_2 **Length** 656 (1 .. 656)

2

1

NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence
Score = 1192 bits (620), Expect = 0.0
Identities = 636/639 (99%), Gaps = 2/639 (0%)
Strand = Plus / Minus

Query: 22 gaaagtcgacatactgtgattggcttagcaaggaagaggaacaatgaagctacc-ataaa 80

Sbjct: 656 gaaagtcgacatactg-gattggcttagcaaggaagaggaacaatgaagctacccataaa 598

Query: 81 taatattatttacacgacagaaaataattggatcgaaaaaatagtagaatttgaaccgaa 140

Sbjct: 597 taatattatttacacgacagaaaataattggatcgaaaaaatagtagaatttgaaccgaa 538

Query: 141 tattattattaacactattgcttgctatggaagacataacgaacctgcaacagctttaat 200

Sbjct: 537 tattattattaacactattgcttgctatggaagatataacgaacctgcaacagctttaat 478

Query: 201 agaaagcaatattcttatgcctatcagagtattagaatctatctcatcacttgatgcagt 260

Sbjct: 477 agaaagcaatattcttatgcctatcagagtattagaatctatctcatcacttgatgcagt 418

Query: 261 attcataaattgtggaacatcactgccaccaaatacgagtttatatgcatatactaaaca 320

Sbjct: 417 attcataaattgtggaacatcactgccaccaaatacgagtttatatgcatatactaaaca 358

Query: 321 aaaagcaaatgaactcgccgccgccattatagataaagtttgtggtaaatatatagagtt 380

Sbjct: 357 aaaagcaaatgaactcgccgccgccattatagataaagtttgtggtaaatatatagagtt 298

Query: 381 aaaattggagcatttctatggagcttttgatggagacgataagtttaccagtatggttat 440

Sbjct: 297 aaaattggagcatttctatggagcttttgatggagacgataagtttaccagtatggttat 238

Query: 441 tagacgttgtttaagtaaccagccagtaaagttaacatctggtttgcaacagagagattt 500

Sbjct: 237 tagacgttgtttaagtaaccagccagtaaagttaacatctggtttgcaacagagagattt 178

Query: 501 cttgtatataaaagatctactaacagcgttcgattgtattataaataatgttaataattt 560

Sbjct: 177 cttgtatataaaagatctactaacagcgttcgattgtattataaataatgttaataattt 118

Query: 561 ccccaaatttcatagtattgaagttggtagtggaaagactatatcaattcgtgaatatgt 620

Sbjct: 117 ccccaaatttcatagtattgaagttggtagtggaaagactatatcaattcgtgaatatgt 58

Query: 621 agagactgttaaaaatatcacaaaaagcaattctataat 659

Sbjct: 57 agagactgttaaaaatatcacaaaaagcaattctataat 19
CPU time: 0.01 user secs. 0.01 sys. secs 0.02 total secs.

Lambda K H
1.33 0.621 1.12

Gapped
Lambda K H
1.33 0.621 1.12

Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 1
Number of Hits to DB: 168
Number of extensions: 7
Number of successful extensions: 5
Number of sequences better than 10.0: 1

Number of HSP's better than 10.0 without gapping: 1
Number of HSP's gapped: 3
Number of HSP's successfully gapped: 1
Number of extra gapped extensions for HSPs above 10.0: 0
Length of query: 721
Length of database: 14,588,094,788
Length adjustment: 26
Effective length of query: 695
Effective length of database: 14,588,094,762
Effective search space: 10138725859590
Effective search space used: 10138725859590
Neighboring words threshold: 0
Window for multiple hits: 0
X1: 11 (21.1 bits)
X2: 26 (50.0 bits)
X3: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)

Discussion

5. DISCUSSION

The primary motivation for controlling *Salmonella* infections in poultry had been reduction of disease losses, and this had led to the implementation of extensive testing programs. To implement such a practice, knowledge of infection levels in flocks is very essential. This would require the development of methods for detection of flock infection on practical and economical basis (Bhatia and McNabb 1980).

Identification and characterization of *Salmonella* by conventional culture methods have been supported by modern molecular biology techniques, which are based on the genetic information of the organism. Molecular approaches such as amplification of specific nucleic acid sequences of the genome have allowed bacterial detection and disease diagnosis, easy, fast and reliable, as compared to the laborious and time consuming conventional techniques. Moreover the greater advantage of these techniques is that it can be applied to mixed microbial specimens without prior isolation of individual species of bacteria.

5.1 ISOLATION OF *SALMONELLA*

Numerous techniques and methods have been described for the isolation of salmonellae from different types of specimens. It is not possible to recommend any particular technique because of the lack of comparative evidence on the efficacy of the isolation methods in current use.

In the present study, clinical samples from dead/ailing and healthy birds were used for detection of *Salmonella.* Environmental samples like hatcher tray swab, droppings, drag swab, feed, water, setter tray swab and egg surface swab were also taken. Similar type of samples were used for isolation of *salmonella* by various authors (Bhatia and McNabb, 1980; Poppe *et al.*, 1991; Batabyal *et al.*, 2002).

Non-selective and/or selective enrichment combined with PCR have been applied for the detection of many bacterial pathogens to improve sensitivity and dilute PCR-inhibitory substances (Schrank *et al.*, 2001). In our present study, RV broth was used as an enrichment broth, in which samples like cloacal swabs, droppings, drag swab, feed, hatcher tray swab, water, setter tray swab and egg surface swab were taken as described by Schrank *et al.* (2001).

In the present study out of the 155 birds screened for *Salmonella*, samples from 15 birds only were showing colonies suggestive of *Salmonella.* None of the environmental samples have shown colonies suggestive of *Salmonella.*

Purushothaman *et al.* (1996) have isolated *Salmonella* from cloacal swabs, poultry litter, fishmeal and caecal swabs of poultry. Batabyal *et al.* (2002) could also isolate *Salmonella* from samples like liver and intestine. Muniyellappa *et al.* (2003) had isolated *Salmonella* from liver samples.

5.2 IDENTIFICATION

Altogether 15 isolates of *Salmonella* were obtained from poultry. All isolates were Gram-negative bacilli. They were non motile, grew aerobically. All were catalase positive, oxidase negative and fermented glucose.

In the second stage biochemical tests, all the isolates tested were indole negative, methyl red positive and Voges-Proskauer negative, urease negative, produced H_2S, reduced nitrate, ornithine decarboxylase positive and citrate utilization negative. These findings are in accordance with the results obtained by Chakraborthy *et al.* (1999) and Batabyal *et al.* (2002).

With regard to the fermentation of the sugars all isolates fermented dulcitol, maltose, arabinose, trehalose, and mannitol. They could not utilize sucrose, cellobiose, and lactose. Chakraborthy *et al.* (1999) also recorded cent per cent fermentation of sugars like dulcitol, maltose, arabinose, trehalose, and mannitol.

On the basis of cultural and biochemical properties all the chicken isolates as well as quail isolates were identified as *Salmonella gallinarum.* This indicated that though the isolates were from different types of birds and samples were from different sources, they all belonged to the same serotype, viz., *Salmonella gallinarum*.

Salmonella gallinarum infection primarily has been considered as a disease of adult birds: but it was revealed from present study, that infection occurs in chicks also, indicating the change of epidemiological patterns of the disease. Chakraborthy *et al.* (1999) could also isolate *Salmonella gallinarum* from chicks of different weeks of age.

5.3 ANTIBIOGRAM

A variety of chemotherapeutic agents have been used in the treatment of salmonellosis. Since there is often a wide variation in the responsiveness of *Salmonella* to these agents, *in vitro* drug sensitivity testing is essential for the selection of an appropriate drug in a given situation.

All the chicken isolates as well as quail isolates were subjected to antibiotic sensitivity testing. All isolates were sensitive to enrofloxacin, pefloxacin, chloramphenicol and ciprofloxacin. These findings are in accordance with those of the other workers (Sujata *et al.*, 2003; Muniyellapa *et al.*, 2003).

Twelve isolates representing 80.00 per cent of the isolates tested were found to be sensitive to ampicillin and amoxycillin , 86.66 per cent isolates tested were found to be sensitive to co-trimoxazole. Similar results have been reported by Chakraborthy *et al.* (1999)

All the isolates were resistant to .tetracycline, furazolidone and cloxacillin. These findings are in accordance with Saikia *et al.* (2002) and Sujata *et al.* (2003).

5.4 SEROTYPING

Six isolates were sent to National Salmonella and Escherichia Center Kasauli for serotyping. Two isolates were serotyped as *Salmonella gallinarum.*

Chakraborthy *et al.* (1999) have reported that serotype *Salmonella gallinarum* was responsible for fowl typhoid in West Bengal. Similar types of findings have also been made by Batabyal *et al.* (2002).

5.5 POLYMERASE CHAIN REACTION FOR THE DETECTION OF GENUS *SALMONELLA*

In recent years, diagnostic laboratories have been concerned with reducing the time required for diagnosis of *Salmonella* infections. The current standard laboratory procedure to culture and identify *Salmonella* serovars takes approximately four to seven days. In addition, *Salmonella* serovars are not detectable in certain clinical samples that contain small numbers of organism. Therefore, a rapid and sensitive method for identification of *Salmonella* from clinical specimens is needed (Park *et al.*, 2001).

Amplification of DNA using PCR can be accomplished rapidly and is of particular value when concentrations of bacteria are low, when bacteria that are shed are non-viable, or when isolation of an organism is difficult. The PCR can be used as a highly sensitive and specific test for the presence of pathogenic bacteria in clinical specimens. Also tests, based on PCR are rapid, reliable, and cost-effective than tradional culture methods.

In the present study, 15 isolates were detected by standard microbiological techniques (SMT). Forty-six clinical samples were found to be positive by RV– PCR with genus specific *invA* primers. The expected 284 bp level amplification was obtained in all 15 isolates. No amplification products were detected when *invA* primers were used to amplify the DNA prepared from

other bacteria like *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*, thereby indicating the specificity of the primers.

The RV –PCR assay thus detected more positive samples than SMT, considerably decreasing the number of false negative results, which commonly occur in diagnostic laboratories. These findings are in accordance with the results obtained by Oliveira *et al.* (2002).

5.6 POLYMERASE CHAIN REACTION USING SEROVAR SPECIFIC PRIMERS

In the present study, those samples (46 clinical samples and 15 *Salmonella* isolates) which were positive by genus level PCR were subjected to a second set of *sefA* primers, to detect *Salmonella enteritidis*, *Salmonella gallinarum* or *Salmonella pullorum* at the serovar level.

All the samples which were found to be positive by genus specific PCR were also positive by serovar specific PCR using *sefA* primers. Similar findings have been made by Oliveira *et al.* (2002).

PCR utilizing *sef* primers was able to detect more number of *Salmonella* positive samples at serovar level when compared to SMT.

5.7 POLYMERASE CHAIN REACTION FOR DETECTION OF *rfb*S GENE OF AVIAN *SALMONELLA*

Recently, polymerase chain reaction has been shown to offer a new strategy in the detection of *Salmonella* using *rfbS* gene. This method is based on the designed primer sets targeting variable regions of the DNA sequences of the *rfbS* gene clusters that are involved in biosynthesis of *Salmonella* lipopolysaccharide (LPS) 'O' antigens.

The 'O' antigen is highly polymorphic and the variation is thought to be of importance in the pathogenesis of many bacteria including *Salmonella*. Many *Salmonella* from groups A, B, or D, have similar O- antigen structures. Routinely, the *rfbS* gene has been used for differentiation of *Salmonella* serotype: serogroup A, B, C1, C2 and D. But it has not been determined whether each *Salmonella* serogroup has different *rfb* gene. Also, differentiation method for each biotype using *rfbS* gene has not been developed respectively (Park *et al.*, 2001).

In the present study by using *rfbS* gene specific primers, the 754 bp amplicon could be obtained from template DNA prepared from direct bacterial culture lysates and isolations made from RV broth.

All the above three PCR protocols were sensitive than SMT that significantly decreases the number of false negative results, often occurring when poultry derived samples are analyzed.

5.8 RESTRICTION ENZYME ANALYSIS OF *rfb*S GENE PCR PRODUCT

Based on identified nucleotide sequence of each *rfb*S gene, restriction enzyme sites were selected using online software for restriction mapping nucleotide sequences (Web cutter, Ver.2.0). As a result, the restriction enzyme sites were found in the nucleotide sequences of *rfb*S genes of *Salmonella gallinarum* (nucleotide position 235 bp) and *Salmonella pullorum* (nucleotide position 239 bp). These enzymes were *Tfi* I for *Salmonella gallinarum* and *Ple* I for *Salmonella pullorum* (Park *et al.*, 2001).

Therefore, in this study RFLP-PCR was applied to differentiate the *Salmonella* serovars, using the *rfbS* gene restriction enzymes. When the *rfbS* gene amplified product (754 bp) was subjected to *Tfi* I enzyme specific for *Salmonella gallinarum* restriction digestion gave rise to fragments at 520 bp and 235 bp levels. But there was no digestion when the same amplified products were subjected to *Ple* I specific for *Salmonella pullorum*, once again confirming that

all the isolates were *Salmonella gallinarum*. This is in concordance with the findings of Park *et al.* (2001).

5.9 PLASMID PROFILE OF *SALMONELLA GALLINARUM* ISOLATES

All the 15 isolates harbored plasmids. Isolate PS1 which harbored four plasmids (48.06 kbp, 5.12 kbp and two low molecular weight bands above 3.36 kbp) was categorized in profile I while the rest of the isolates, each of which carried a single high molecular weight plasmid (48.06 kbp) and an low molecular weight plasmid (above 3.36 kbp) were grouped in profile II. Mary (2000) reported that analysis of five avian salmonellae revealed diverse characters with respect to the number and molecular size of the plasmids. The number of plasmids varied from two to three with molecular size ranging from 1.75 kbp to 48.32kbp and she could identify two identical low molecular weight plasmids in *Salmonella gallinarum*.

5.10 MULTIPLEX PCR FOR VIRULENCE PLASMID

There are six *Salmonella* serovars known to contain the virulence plasmid carrying *spvC* genes: *S. typhimurium*, *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum* and *S. pullorum*. Except for *S. gallinarum* and *S. pullorum*, which are specific for fowl, the other serovars named here are common etiologic agents of enteritis in humans. Therefore, the appearance of at least one band, or two bands if there was a virulence plasmid, would indicate the presence of *Salmonella* (Jenikova *et al.*, 2000).

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 etiological agent. In the present study the expected 571 bp level amplification, which is specific for *Spv* virulence region and 284 bp level amplification, which is specific for genus *Salmonella* were obtained in all the isolates.

5.11 ALLELE SPECIFIC PCR FOR *SALMONELLA GALLINARUM*

Serotypes *Salmonella gallinarum* (SG), *Salmonella pullorum* (SP) and *Salmonella enteritidis* (SE) are very similar from the point of view of their antigenic structure. However, the differentiation between SG and SP requires further sub-typing of 'O' antigen subfactors or use of classical methods based on fermentation of maltose, dulcitol, rhamnose, ornithine decarboxylase and utilization of d-tartarate. Some of these assays are tedious, time consuming (normally taking 5 to 7 days for definitive diagnosis) and infrequently performed by veterinary diagnostic laboratories. Moreover, recent reports of intermediate strains with variable biochemical pattern casts doubts on the validity of these biochemical assays (Shah *et al.,* 2004).

Cloning and sequence analysis of *rfb*S gene identified 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 (Shah *et al.,* 2004).

The expected 187 bp level amplification specific for *Salmonella gallinarum* by using primer pair (*rfb*SF, *rfb*SG) was detected in all the 15 isolates employed in this study. None of the isolates gave 187 bp level amplification, specific for *Salmonella pullorum* when primer pair (*rfb*SF, *rfb*SP) specific for *Salmonella pullorum* was used. These findings are in accordance with results obtained by Shah *et al.* (2004).

5.12 GENE SEQUENCING OF *rfbS* PCR AMPLICON

The PCR product with an approximate molecular size of 754 bp, sequenced by Sanger's dideoxy chain termination method revealed a product of

656 base pairs. The sequence has been submitted to the Genbank and has been assigned the accession No AF 442573 ATCC 9184.

Sequence similarity searches were performed with BLAST provided by NCBI showed a 99 per cent identity with *Salmonella gallinarum*. These findings are in accordance with Park *et al.* (2001). The PCR products were purified and directly sequenced. Their study revealed a high degree of homology among different serotypes.

The present study indicates that serotype *Salmonella gallinarum* is prevalent in Kerala. However, continuous monitoring of the field situation for emergence of new serotypes is a must for effective control of the disease. This situation necessitates the serotyping of the field isolates at the earliest. The PCR-RFLP, as well as allele specific PCR offer a simple effective tool in this endeavor. This technique has the potential to identify a serotype within a few hours of the receipt of the sample.

The results indicated a greater efficacy of RV broth as an effective enrichment media for *Salmonella* isolation. The PCR technique coupled with culture in an enrichment broth could distinguish *Salmonella* species from other enteric bacteria when present in samples from the poultry. This method can be applied to different kinds of samples from the poultry sector. This study demonstrates the higher sensitivity of the PCR with particular relevance to the poultry sector in the evaluation of low levels of infection and/or contamination by *Salmonella*. Therefore, the PCR technique would be the best choice for *Salmonella* monitoring whenever stringent biosecurity programmes are being applied.

Summary

6. SUMMARY

The parameters included in the present investigation were detection of salmonellae in clinical samples by *inv* PCR, *sef* PCR and *rfbS* gene specific PCR. The isolates of *Salmonella* were characterized by *inv* PCR, *sef* PCR, *rfbS* gene specific PCR, mutiplex PCR and allele specific PCR. The isolates were also subjected to restriction enzyme analysis of *rfbS* gene. Plasmid profiles of the isolates was carried out. The *rfbS* PCR amplicon was sequenced.

Isolation of *Salmonella* was attempted from 155 birds and thirty-four environmental samples. Altogether 15 isolates, 13 from chicken and two from quails were obtained. All the isolates have been characterized as *Salmonella gallinarum* by morphological, cultural and biochemical tests.

In biochemical characterization, 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.

With regard to the fermentation of the sugars all isolates fermented dulcitol, maltose, arabinose, trehalose, and mannitol. They could not utilize sucrose, cellobiose and lactose. Xylose could be fermented only by isolates PS 1, 2, 3, 5, 6, 13 and QS1, QS2. Sorbitol was not utilized by two isolates, one each from chicken and quail.

All isolates were uniformly sensitive to chloramphenicol, ciprofloxacin, enrofloxacin, and pefloxacin. All were resistant to tetracycline, furazolidone and cloxacillin.

Out of 15 isolates, two isolates were serotyped as *Salmonella gallinarum* by the National Salmonella and Escherichia Center, Kasauli.

Out of the 155 samples subjected to Rappaport-Vassiladis polymerase chain reaction (RV-PCR) 46 samples were positive by both genus specific as well

as serovar specific PCR. From the PCR positive samples, 15 isolations could be made by standard microbiological techniques (SMT).

All the 15 isolates when subjected to an third PCR, specific for *rfbS* gene, an expected 754 bp amplified product had been obtained. Amplicon from the *rfbS* gene was then subjected for restriction enzyme analysis by using two types of restriction enzymes namely *Tfi* I specific for *Salmonella gallinarum* and *Ple* I specific for *Salmonella pullorum.* All the 15 isolates were uniformly digested by *Tfi* I, while none of the isolates were digested by *Ple* I

Plasmid profile analysis was carried out. The number of plasmids varied from two to four, with molecular size ranging from 3.36 kbp to 48.06 kbp.

A multiplex PCR for virulence plasmid was carried out. It could simultaneously identify the *Salmonella* strains and the presence of virulence plasmid, thus facilitating the search for specific etiological agent. In the present study the expected 571 bp level amplification, which is specific for *Spv* virulence region and 284 bp level amplification, which is specific for genus *Salmonella* were obtained in all the isolates

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. The expected 187 bp level amplifications were obtained in all the isolates.

The PCR product with an approximate molecular size of 754 bp, sequenced by Sanger's dideoxy chain termination method revealed a product of 656 base pairs. The sequence has been submitted to the Genbank and has been assigned the accession No AF 442573 ATCC 9184. Sequence similarity searches performed with BLAST provided by NCBI showed a 99 per cent identity with *Salmonella gallinarum*.

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NUCLEIC ACID BASED DETECTION OF SALMONELLAE IN POULTRY

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ABSTRACT

In the present study detection of salmonellae by Polymerase Chain Reaction in avian bio-materials was carried out. Isolation of salmonellae from avian bio-materials was also done. Differentiation of salmonellae based on molecular methods was carried out.

Thirteen isolates from chicken and two from quails were characterized as *Salmonella gallinarum* using standard bacteriological procedures. With regard to the fermentation of the sugars all isolates fermented dulcitol, maltose, arabinose, trehalose, and mannitol. Variation in fermentation pattern was observed with xylose and sorbitol. All isolates were uniformly sensitive to chloramphenicol, ciprofloxacin, enrofloxacin, and pefloxacin, while all were resistant to tetracycline, furazolidone and cloxacillin. Two isolates were serotyped as *Salmonella gallinarum* by the National Salmonella and Escherichia Center, Kasauli. Forty-six samples were positive by both genus specific as well as serovar specific PCR.

The genus specific and serovar specific PCR were used to confirm the identity of the isolates. Performing PCR on template DNA prepared from RV broth enriched sample was found to be an extremely rapid method for detection of *Salmonella*. Restriction enzyme analysis of the amplicon from the *rfbS* gene with enzyme *Tfi* I of all isolates revealed the expected 235 bp digestion.

All the isolates carried plasmids. Two plasmid profiles were observed among the isolates examined. A multiplex PCR for virulence plasmid was carried out. The expected 571 bp level amplification, which is specific for *Spv* virulence region and 284 bp level amplification, which is specific for genus *Salmonella,* were obtained in all the isolates. An allele-specific PCR method was developed for serotype-specific detection of *S. gallinarum*. The expected 187 bp level amplicons were obtained in all the isolates.

The sequence of the *rfbS* gene product has been submitted to the Genbank and has been assigned the accession No AF 442573 ATCC 9184 . The sequence showed 99 per cent identity with *Salmonella gallinarum.*