MOLECULAR CHARACTERISATION OF SALMONELLAE ISOLATED FROM POULTRY

By

G. TRESSA MARY

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"MOLECULAR CHARACTERISATION OF SALMONELLAE ISOLATED FROM 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.

Tressa Mary

G. TRESSA MARY

Mannuthy

20.1.2000

CERTIFICATE

Certified that the thesis, entitled **"MOLECULAR CHARACTERISATION OF SALMONELLAE ISOLATED FROM POULTRY"** is a record of research work done independently by **Ms. G. Tressa Mary**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

20/1/2000

Dr. K.T. Punnoose
(Chairman, Advisory Committee)
Professor and Head
Department of Microbiology
College of Veterinary and
Animal Sciences, Mannuthy

Mannuthy
20th Jan. 2000

CERTIFICATE

We, the undersigned members of the Advisory committee of **Ms. G. Tressa Mary,** a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled **"MOLECULAR CHARACTERISATION OF SALMONELLAE ISOLATED FROM POULTRY"** may be submitted by Ms. G. Tressa Mary, in partial fulfilment of the requirement for the degree.

27/1/2000

Dr. K.T. Punnoose
(Chairman, Advisory Committee)
Professor and Head
Department of Microbiology
College of Veterinary and Animal Sciences, Mannuthy

Dr. V. Jayaprakasan
Associate Professor
Department of Microbiology
(Member)

MINI

Dr. M. Mini
Assistant Professor
Department of Microbiology
(Member)

Dr. K.V. Reghunandanan
Associate Professor
Centre for Advanced Studies in Animal Genetics and Breeding
(Member)

28/1/2000

External Examiner
DR. T. G. PRABHAKAR

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G. Tressa Mary

Mannuthy

To my
appa and amma

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ABBREVIATIONS

A	-	ampicillin
AGE	-	agarose gel electrophoresis
AO	-	acridine orange
BGA	-	Brilliant Green agar
C	-	chloramphenicol
CCC	-	covalently closed circular
Cf	-	ciprofloxacin
Ch	-	cephalothin
Cm	-	cotrimazine
Cx	-	cloxacillin
D	-	doxycycline
DNA	-	Deoxyribonucleic acid
EB	-	ethidium bromide
EDTA	-	Ethylene diamine tetra acetate
Ex	-	enrofloxacin
F	-	furazolidone
G	-	gentamicin
HMW	-	high molecular weight
K	-	kanamicin
kb	-	kilobase
LB	-	Luria bertani
MA	-	micro agglutination
MCA	-	Mac Conkey
Mdal	-	megadalton
µg	-	microgram
MHA	-	Mueller Hinton agar
N	-	neomycin
Na	-	nalidixic acid
Nf	-	nitrofurantoin

O	-	oxytetracycline
ONPG	-	O-nitrophenyl-ß-D-galactopyranoside
PBS	-	phosphate buffered saline
R	-	rifampicin
RE	-	restriction enzymes
REA	-	restriction enzyme analysis
REAC	-	restriction enzyme analysis of chromosomal DNA
S	-	streptomycin
SDS	-	sodium dodecyl sulphate
SDS-NaOH	-	sodium dodecyl sulphate-sodium hydroxide
S_3	-	triple sulpha
Sz	-	sulfadiazine
T	-	tetracycline
TBE	-	Tris borate electrophoresis
TE	-	Tris EDTA
TEG	-	Tris EDTA glucose
Tr	-	trimethoprim
TSI	-	Triple sugar iron agar
UV	-	Ultra violet

LABORATORY REFERENCES

SB	-	*Salmonella branderup*
SE	-	*S. enteritidis*
SG1	-	*S. gallinarum*
SG2	-	*S. gallinarum*
ST	-	*S. typhimurium*

Introduction

1. INTRODUCTION

Avian salmonellosis is a scourge of poultry industry causing heavy economic loss and is responsible for food borne infections in human beings. Fowl typhoid caused by *Salmonella gallinarum*, bacillary white diarrhoea caused by *S. pullorum* and paratyphoid infections caused by motile salmonellae are the most important infections seen in birds. Infections by motile salmonellae in poultry are generally subclinical and are responsible for food borne infections. Heavy mortality in young chicks, chronicity, carrier status in adult birds and transovarian transmission lead to termination of breeding operations and shut down of hatchery units. Many serovars of salmonellae have been identified in domestic poultry, among them one serovar may be a predominant isolate for a number of years before it is replaced by another. Probably all salmonellae could transiently infect poultry and thus constitute a potential health hazard. So maintenance of a flock free of salmonella is warranted for economic viability of the poultry industry and for safeguarding human beings from food borne infections.

A host of methodologies have been developed to identify relatedness of salmonella isolates in epidemiological investigations. Phenotypic methods include antimicrobial susceptibility testing, biotyping, colicin typing and phage typing. These methods have been supplemented by genotypic characterisation methods such as plasmid profile analysis, analysis of restriction endonuclease cleavage pattern of both plasmid and genomic DNA.

The number and molecular weight of plasmids which compose the plasmid profile is a preferable method to differentiate strains from one another and has proven to be a very discriminating tool within the same serotypes. A further degree of differentiation can be achieved by digesting the plasmids with type II restriction endonucleases or cutting enzymes and analysing the fragments after agarose gel electrophoresis.

Current evidence has indicated that high molecular weight (HMW) plasmids are necessary for the virulence of several serotypes of salmonella which are capable of producing disease in animals and human beings. These HMW plasmids have been shown to be present in *S. typhimurium*, *S. dublin*, *S. enteritidis*, *S. gallinarum* and *S. pullorum*. The loss of these plasmids have been shown to result in a loss of virulence in these serotypes (Bichler *et al.*, 1994).

In salmonellae that do not carry plasmids or possess only serotype specific plasmids, it may be necessary to use chromosomally based methods for molecular characterisation. Restriction enzyme analysis of chromosomal DNA (REAC) may be used as a simple and rapid method since it permits a direct approach to differentiate organisms whether or not alternative typing methods are available.

This study is therefore undertaken to characterise the salmonellae of avian origin based on

1. Identification of salmonellae by morphological, cultural, biochemical characters and serotyping.

2. Testing the antimicrobial sensitivity pattern.

3. Examination of the number and size of plasmids which compose the plasmid profile.

4. Analysis of restriction endonuclease cleavage pattern of plasmid DNA.

5. Elimination or curing of plasmids by physical and chemical agents.

6. Conduct of virulence studies in day old chicks using wild and cured isolates.

7. Analysis of restriction endonuclease cleavage pattern of chromosomal DNA for genomic relatedness.

8. Assessment of relationship between these properties.

Review of Literature

2. REVIEW OF LITERATURE

2.1 History and nomenclature of salmonella

Salmon and Smith, 1885 isolated a bacterium for the first time from pigs which had died of hog cholera and named it as *Bacillus choleraesuis*, believing it to be the cause of the disease. Gartner, 1888 isolated a similar organism of this group from a human patient suffering from gastroenteritis and later it was named as *Salmonella enteritidis*. It was only in 1900 that the name *Salmonella* was given to this group of organisms by Ligniers in honour of D.E. Salmon, the pioneer American Bacteriologist (Brandly *et al.*, 1966).

The terminology introduced by early workers accorded specific rank to each antigenically distinguishable type and later it was established that each new type should be named after the place from which it was isolated. The current number of serotypes is 2399 (Popoff *et al.*, 1995). The first published Kauffmann-White table contained some 20 serotypes, each considered to be a species, but they should no longer be accorded specific species status (LeMinor *et al.*, 1982; Popoff *et al.*, 1992).

The Genus *Salmonella* is now considered to comprise two species, *S. enterica* and *S. bongori*. There are six subspecies in *S. enterica*, the most important of which is *S. enterica* subsp. *enterica* which includes the typhoid and paratyphoid bacilli and most of the serotypes responsible for wide spread disease

in mammals like *S. typhimurium*, *S. enteritidis*, *S. dublin*, *S. gallinarum* and *S. pullorum*. The other subspecies are *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. *Salmonella enterica* subsp. *enterica* serovar Typhimurium can be designated as *Salmonella* ser. Typhimurium or *Salmonella* Typhimurium or simply Typhimurium for use in clinical situations and indicate further that the named serotype is a member of subsp. *enterica* (Old and Threlfall, 1998).

2.1.1 Avian salmonellosis

The domestic poultry constitutes the largest single reservoir of salmonella organisms (Hofstad *et al.*, 1972).

2.1.2 Nonmotile host adapted serotype

In the *S. enterica* subsp. *enterica* ser. Gallinarum, two distinct biovars are described, viz., Gallinarum and Pullorum (Snoeyenbos, 1984). Bioserotype Gallinarum, first described by Klein, 1889 is the agent causing fowl typhoid in both adult and young chickens and in other birds such as Turkeys. Bioserotype Pullorum, first isolated by Rettger in 1900 is the causative agent of acute diarrhoeal disease in chicks. Adult birds may act as carriers and spread the infection by transovarian transmission.

2.2.3 Motile non host adapted serotypes

The infections of poultry by motile salmonellae are called paratyphoid infections (Williams, 1978). *S. enterica* ser. Typhimurium was the most prevalent

serotype in intensively reared poultry world wide until the emergence of infection by *S. enterica* serovar Enteritidis (Rodriquez *et al.*, 1990).

2.2 Identification

Salmonellae were identified by morphological, cultural and biochemical tests (Holt *et al.*, 1994).

2.3 Serotyping

The antigenic formula consists of three parts, the somatic '0' antigen, the phase I H antigen and the phase II H antigen (Kauffmann, 1966).

Gupta and Verma (1991) developed a micro agglutination (MA) test for serotyping salmonella in view of saving antigens/antisera and glasswares. MA can replace slide agglutination test and tube agglutination test in serodiagnosis of certain salmonella infections.

2.3.1 Kauffmann white diagnostic scheme

Only antigens of diagnostic value are cited in this scheme. A simplified version of the scheme based on only 12 O, 18 H sera and Vi serum gives sufficient information for routine diagnostic identifications (Ewing, 1986).

2.4 Antibiogram

The term resistance and susceptibility were used in antibiotic sensitivity tests to express the ability or lack of ability, of an organism to multiply in the

presence of a given concentration of an antibiotic under defined conditions and it is described as multi resistant if it was resistant to more than one antimicrobial agent.

The drug resistance in enterobacteriaceae was first demonstrated in Japan (Akiba *et al.*, 1960).

Antibiotic resistance could be assessed by using various antibiotics at critical concentrations by agar dilution, broth dilution and the agar diffusion test (Barry, 1976). The various patterns of antibiogram could be capable of distinguishing between strains of bacteria.

The genes coding for resistance may be present either in chromosome or plasmids or transposons (Dax, 1997).

Boachie (1985) found that all the 29 isolates from poultry were sensitive to furazolidone. All but one were sensitive to chloramphenicol and 23 to neomycin but nearly 50 per cent were resistant to oxytetracycline, chlortetracycline and streptomycin.

Saxena *et al.* (1985) emphasised the increased prevalence of resistant strains of salmonellae to ampicillin, chloramphenicol and furazolidone in India.

Poppe and Gyles (1987) observed that 54 out of 172 salmonellae from avian sources were resistant to atleast one drug and multiple drug resistance was associated with R plasmids and transmissible plasmids that encoded resistance to chloramphenicol and gentamicin.

Multiple drug resistant strains of *S. typhimurium* carried two plasmids, one of which is 5.5 Mdal weight that coded SSu resistance was common to all strains. The second plasmid was of approximately 70 Mdal in 16 strains of R-type ACGKSSuTTm, 67 Mdal in five strains of R-type CGKSSuTTm, 65 Mdal in three strains of R-type ACSSuT and 60 Mdal in one strain of R-type CSSuT (Threlfall *et al.*, 1989).

Mathew *et al.* (1991) noted that the *S. branderup* isolated from egg was sensitive to chloramphenicol, norfloxacin, gentamicin, polymixin, kanamicin, ampicillin, cefazolin, carbenicillin, septran and furazolidone and resistant to tetracycline, doxycycline and erythromycin.

On analysis of *S. enteritidis* strains, Poppe *et al.* (1993) reported that 17 per cent were resistant to one or more of the anti microbial drugs. Resistance to sulfisoxazole was common followed by resistance to nitrofurantoin. Gentamicin resistance was associated with self transmissible plasmid of 60 Mdal.

Oh and Choi (1994) performed antibiogram using nine antibiotics ampicillin, chloramphenicol, gentamicin kanamicin, nalidixic acid, rifamipicin, streptomycin, sulfa dimethoxine and tetracycline on various salmonella serotypes isolated from chicks and reported that all the strains were sensitive to rifampicin but 23.8 per cent were resistant to one or more drugs.

Multiple drug resistant *Salmonella enteritidis* isolates were more from non human sources than human sources (Nair *et al.*, 1995).

Blaszczak *et al.* (1996) observed increasing percentage of resistance to neomycin (16.0 and 26.2) terramycin (4.8 and 71.9), enrofloxacin (6.1 and 34.2), streptomycin (40 and 61.4) in 1994 and 1995 respectively among the poultry isolates of salmonella.

Poppe *et al.* (1996) observed a high percentage of resistance to spectinomycin among the salmonella isolates from layer and broiler flocks in Canada.

2.5 Plasmid profile analysis

2.5.1 Bacterial plasmids

Plasmids are covalently closed circular (CCC) double stranded, extrachromosomal, autonomous DNA molecules found in many bacterial cells. They vary in size from approximately one kb to greater than 400 kb and in copy number from one to several hundred per cell. Bacterial plasmids contribute a wide variety of phenotypes to their host, including antibiotic resistance and virulence properties. The five main types are (1) fertility (F) plasmids, (2) Resistance (R) plasmids, (3) Col plasmids, (4) Degradative plasmids, (5) Virulence plasmids (Brown, 1990).

2.5.2 Isolation of plasmid DNA

Most of the techniques employed for the isolation of plasmid DNA are based on their supercoiled CCC configuration within the bacterial cell.

For isolation of plasmid DNA, one usually uses anyone of the following properties, such as (1) difference in base composition from chromosomal DNA, (2) small size relative to the chromosome, (3) transferability, (4) circularity. Many methods have been described for the isolation of plasmid DNA. These methods involve three basic steps (1) Growth of the bacteria, (2) Harvesting and lysis of the bacteria, (3) Purification of plasmid DNA (Sambrook *et al.*, 1989).

2.5.2.1 Alkaline extraction method

The "clear lysate method" with the incorporation of Brij, a detergent was widely used (Clewell and Helinski, 1969).

Guerry *et al.* (1973) evolved a procedure in which the plasmid DNA ranging from 5×10^6 to 65×10^6 daltons may be separated from chromosomal DNA by the preferential precipitation of higher molecular weight chromosomal DNA in the presence of SDS and a high concentration of sodium chloride (5 M).

Birnboim and Doly (1979) developed a rapid extraction method of plasmid DNA by utilizing the principle of selective alkaline denaturation of high molecular weight chromosomal DNA wherein a narrow range of pH (12.0 to 12.5) was used to denature the linear DNA but not CCC DNA and that this property could be used for purifying CCC DNA.

Kado and Liu (1981) described a procedure for the detection and isolation of plasmids of sizes ranging from 2.6 to 350 Mdal that are harboured in various species of bacteria. This method utilised the molecular characteristics of CCC

DNA that is released from cells under conditions that denatures chromosomal DNA by using alkaline SDS (pH 12.6) at elevated temperature.

Birnboim (1983) confirmed the usefulness of alkaline denaturation method for the isolation of highly purified CCC DNA.

Sambrook *et al.* (1989) made some modification of the method of Birnboim and Doly (1979) in which, the use of lysozyme at the first step was avoided and phenol-chloroform step was taken as optional.

Sikka (1992) described a method of "in gel lysis analysis" of plasmid profile of bacteria. The principle was that the cells grown with selection were mixed with lytic mix and immediately loaded into the wells of SDS-agarose gel.

Rychlik (1996) described a modification of the hot alkaline method of Kado and Liu (1981) in which the SDS was replaced with Triton x 100. The DNA purified by this method was readily digested with restriction endonucleases for a variety of applications.

2.5.2.2 Boiling method

Boiling method involved lysis of cell with detergent and lysozyme followed by boiling (Holmes and Quigley, 1981).

Gomez-Marquez *et al.* (1987) developed a procedure for large scale purification of plasmid DNA by adopting a modified boiling procedure suggested by Holmes and Quigley (1981).

The boiling method is not recommended for small scale preparation of DNA from strains of *E. coli* that express endonuclease A (Sambrook *et al.*, 1989).

2.5.3 Purification of plasmid DNA

Most of the techniques devised for purification of plasmid DNA have developed based on the CCC nature of plasmid DNA and its consequent resistance to denaturation (Helinski and Clewell, 1971).

Centrifugation of lysates of plasmid bearing strains on alkaline sucrose gradients had also been used to purify plasmid DNA (Freifelder, 1968).

Kado and Liu (1981) used phenol chloroform extraction to remove cell debris and proteins from plasmid DNA preparations. Under these conditions, chromosomal DNA concentrations were either reduced or eliminated.

Gomez-Marquez *et al.* (1987) described a procedure which involved extraction of plasmid DNA initially with phenol chloroform, secondly with chloroform isoamyl alcohol that was followed by ethanol precipitation. The final step was gel filtration chromatography employing S-Sephacryl S-1000.

Sambrook *et al.* (1989) suggested the use of ion exchange or gel filtration chromatography or differential precipitation to separate plasmid and host DNA. Differential precipitation with polyethylene glycol yielded plasmid DNA of extremely high purity.

Sullivan and Klaenhammer (1993) evolved a procedure for the isolation of high quality DNA in which after alkaline extraction, the pellet was resuspended in 7.5 M ammonium acetate containing ethidium bromide and further purified by phenol-chloroform treatment followed by ethanol precipitation.

2.5.4 Precipitation with ethanol

The most widely used method for concentrating DNA is precipitation with ethanol. The precipitate of DNA which was allowed to form at low temperature (-20°C or less) in the presence of moderate concentrations of monovalent cations and was recovered by centrifugation and redissolved in an appropriate buffer at the desired concentration. The technique was rapid and was quantitative even for nanogram amounts of DNA (Maniatis *et al.*, 1982).

2.5.5 Purity of plasmid DNA

The purity of plasmid DNA, prepared was tested by UV spectrophotometric determination.

DNA fractions were deemed to be acceptable when the ratios of absorbance at 260 nm and 280 nm (A260/A280) were greater than or equal to 1.8 and the ratios of absorbance at 260 nm and 230 nm (A260/230) were greater than or equal to 1.5 (Orgam *et al.*, 1987).

The shape of the UV absorption spectra obtained was compared with published data and the purity of the preparation was commented. The extinction

values at 260 nm and 280 nm were used to detect protein contamination and the E260/E280 ratios should be between 1.8 and 1.9 for pure DNA and a value less than 1.75 indicated significant protein contamination (Plummer, 1988).

2.5.6 Agarose gel electrophoresis of plasmid DNA

The utility of agarose gel electrophoresis in the analysis of plasmid DNA molecules had been well established (Meyers *et al.*, 1976; Willshaw *et al.*, 1979; Maniatis *et al.*, 1982 and Sambrook *et al.*, 1989).

2.5.7 Molecular size estimation of plasmid DNA

Molecular size of plasmid DNA was estimated by the inclusion of plasmid molecules of known molecular size in the same electrophoretic run.

Meyers *et al.* (1976) have shown that there was a linear relationship between the logarithms of relative migration of CCC DNA molecules and the logarithm of the plasmid molecular size. The contour length of plasmid was determined by electron microscopy. Molecular weights were calculated from the contour lengths by using the conversion factor of 1 μm = 2.07×10^6 (Meyers *et al.*, 1976).

A strain of *E. coli* V517 which contains eight plasmid species ranging in size 1.36×10^6 to 35.8×10^6 daltons could be employed as reference in agarose gel electrophoresis as a single source of CCC DNA molecules of different sizes (Macrina *et al.*, 1978).

Several regression methods were tested for estimating the size of a wide range of plasmids (1.37 to 31.2 Mdal) and restriction fragments (2.2 to 14.2 Mdal) by agarose gel electrophoresis. Rochelle *et al.* (1985) evolved a most accurate and least variable method which was the multiple regression of $\log_{10}$ molecular size against $\log_{10}$ relative mobility and the reciprocal square root of the relative mobility. It was suggested that with this method, the molecular size of unknown plasmids could be accurately estimated using the plasmids from *Escherichia coli* V517 and *E. coli* IR713 as standards.

2.6 Plasmid analysis in salmonellae

Jones *et al.* (1982) demonstrated a correlation of autonomous 60 Mdal plasmid to the expression of virulent phenotypes such as adhesiveness and invasiveness which enabled the strains to survive the defense mechanisms of the host.

Terakado *et al.* (1983) isolated *S. dublin* organisms from cattle and demonstrated the association of virulence properties to the plasmid of approximately 50 Mdal.

Baird *et al.* (1985) observed that in certain strains of *S. dublin*, *S. enteritidis* and *S. gallinarum*, the loss of a 55 Mdal, a 36 Mdal and an 51 Mdal plasmid respectively had resulted in loss of virulence. Strains of *S. typhimurium* have been shown to harbour an approximately 60 Mdal plasmid mediating virulence.

Helmuth *et al.* (1985) showed that the carriage of virulence associated plasmids of sizes 60 Mdal, 37 Mdal, 56 Mdal and 30 Mdal in *S. typhimurium*, *S. enteritidis*, *S. dublin* and *S. choleraesuis* respectively. These plasmids are of high molecular weight that may be regarded as serotype specific plasmids which can be very useful in epidemiological studies.

The contribution of large plasmid of 85 kb to the virulence of *S. gallinarum* and to the production of fowl typhoid is well established. Considerable homology appeared to exist between the large plasmids of *S. gallinarum*, *S. pullorum*, *S. typhimurium*, *S. dublin* and *S. enteritidis* (Barrow *et al.*, 1987).

Poppe and Gyles (1987) noted that no serovars from avian sources other than *S. enteritidis*, *S. typhimurium*, *S. heidelberg* carried serovar associated plasmids.

Barrow and Lovell (1988) analysed the plasmid profiles of eight epidemiologically separate strains of *S. pullorum* and found that seven of these strains contained a large of plasmid of 85 kb and one strain possessed two large plasmids and each of the eight strains possessed atleast one smaller plasmid of between 2.5 to 4 kb.

Threlfall *et al.* (1990) suggested the plasmid profile typing as a method of supplementing phage typing of *S. typhimurium*.

Christensen *et al.* (1992) demonstrated six different plasmid profiles within biovar pullorum while only four in biovar gallinarum and suggested that plasmid

profiling could be used as an epidemiological marker in backtracing infections and the differences in biochemical properties and plasmid content indicated that they represented distinct clonal lines.

Atanassova *et al.* (1993) demonstrated serovar specific plasmids of 62 Mdal and 36 Mdal in *S. typhimurium* and *S. enteritidis* respectively.

Plasmid profiling might serve as an effective adjunct to phage typing for sub-division or differentiation between strains if particular plasmid was very common among one strain (Poppe *et al.*, 1993)

Anjanappa *et al.* (1994) carried out the plasmid DNA isolation from 25 field strains of *S. gallinarium* and found that 19 out of 25 carried a single plasmid of 56 Mdal except three strains which had an additional plasmid of 2 Mdal.

Bichler *et al.* (1994) showed specific plasmid profile could be useful in establishing causal relationships.

Christensen *et al.* (1994) observed only one plasmid profile among the 19 strains of serovar Gallinarum and this profile contained a 85 kb virulence plasmid.

Nair *et al.* (1995) evaluated the gross plasmid analysis and indicated that many of isolates from non human sources contained HMW plasmids of size 55 kb than did isolates from human beings.

Singh *et al.* (1996) analysed nine *S. gallinarum* strains and determined the presence of 2.5 kb plasmid in all strains and an additional plasmid of 85 kb in all isolates except one.

On analysis of plasmids in strains of *S. typhimurium* from poultry in India, Rathor *et al.* (1998) found the presence of 85 kb plasmid in all the 12 strains. Besides, a 100 kb plasmid was found in two strains while the other strains had 2 kb and 0.1 kb plasmids.

2.7 Restriction enzyme analysis (REA) of plasmid DNA

2.7.1 Restriction enzymes (RE)

Restriction enzymes are enzymes that cut DNA at specific sequences within the molecule and named after the bacterium from which they are isolated. These DNA cuts result in blunt ends or sticky ends.

EcoRI	GAATTC	*E. coli* RY 13
	CTTAAG	
HindIII	AAGCTT	*Haemophilus influenzae* Rd
	TTCGAA	

2.7.2 REA of plasmid DNA in avian salmonellae

Plasmid profiling and plasmid endonuclease fingerprinting could be used to investigate the molecular epidemiology of antibiotic resistance in salmonellae from animals and humanbeings in U.S. (Bezanson *et al.*, 1983).

Terakado *et al.* (1983) performed the REA of 50 Mdal plasmid isolated from cattle in Japan (20 strains) and in Europe (2 strains) indicated high relatedness among them because the EcoRI and HindIII cleavage patterns independent of geographic source were indistinguishable from each other. They suggested a common ancestry among such 50 Mdal plasmids.

Helmuth *et al.* (1985) recorded the identical restriction fragments of serotype specific plasmids from various salmonella serotypes after digestion with HindIII.

Nakamura *et al.* (1985) concluded that the 36 Mdal plasmids from various *S. enteritidis* strains were derived from the same origin as they revealed identical cleavage patterns with EcoRI, HindIII and BamHI and also suggested that this plasmid was native to *S. enteritidis*.

The sensitivity of the plasmid typing could be increased by cleaving plasmid DNA with a limited number of endonucleases and the resultant fingerprint might be used to discriminate between plasmids of similar molecular weights (Platt *et al.*, 1986).

Existence of three (A, B, C) restriction profiles for 85 kb virulence plasmid in biovar gallinarum and only 2 in biovar pullorum was demonstrated by Christensen *et al.* (1992).

The most rational typing strategy for *S. berta* was the plasmid profiling in combination with REA of plasmids (Olsen *et al.*, 1992).

Dorn *et al.* (1993) documented the egg borne transmission of *S. enteritidis* to humans as evidenced by plasmid rofiling and restriction fragmentation pattern which showed matching of human isolates to chicken isolates.

Christensen *et al.* (1994) demonstrated that the REA of virulence plasmid of 85 kb in Danish isolates of biovar gallinarum belonged to the restriction profile A (Christensen *et al.*, 1992).

Kinde *et al.* (1996) noted the identical plasmid profile and restriction digestion pattern between the *Salmonella enteritidis* isolates of effluent and the resident feral animals and chicken, which enabled them to pinpoint the source of infection in the flock to the effluent.

Christensen *et al.* (1997) performed REA of the 180 kb plasmid to ensure that the plasmids found in the isolates from the broiler flocks and in the isolates from the hatchery were identical. Digestion of plasmids from each source with HindIII generated DNA fragments of identical sizes.

2.8 Curing of salmonella plasmids

Elimination of plasmids by curing is an essential step in procedures used to investigate virulence plasmids.

Chemical agents like acridine dyes, ethidium bromide (EB), sodium dodecyl sulphate (SDS) and novobiocin and physical agent, growth at elevated

temperatures were found to free or "cure" bacterial cells of plasmid DNA molecules (Novick, 1969).

Plasmid molecules which occur as autonomously existing circular DNA complexes could be eliminated by these agents either due to interference with their replication (acridines, EB, novobiocin) or by alterations of their membrane attachment sites (SDS and elevated temperature) (Carlton and Brown, 1981).

Tomoeda *et al.* (1968) observed that the detergent SDS was effective in eliminating F' lac and the R factor from *E. coli*.

Hahn and Ciak (1976) examined 18 DNA complexing compounds at the standard concentration of 10^4 M for the elimination of resistance determinants and found nitroacridine II proved to be most active compound. Fifteen intercalative compounds and nalidixic acid eliminated four antibiotic resistance determinants in *S. typhimurium* with different frequencies. The elimination of plasmids was as a result of selective toxicity for plasmid template DNA and inhibition of R-factor replication.

Carlton and Brown (1981) evolved methods for curing by incorporating the chemicals like acridine, EB, SDS and elevated temperature.

Jones *et al.* (1982) obtained 10^{-2} and 10^{-3} frequencies of curing in *S. typhimurium* strains by treating it with novobiocin, EB or SDS.

Ethidium bromide eliminated 36 Mdal plasmid from *S. enteritidis* (Nakamura *et al.*, 1985).

Barrow *et al.* (1987) obtained cured derivatives of *S. gallinurium* by culturing at 42°C.

Barrow and Lovell (1988) reported the elimination of large molecular mass plasmid from salmonella by passage in nutrient broth containing acridine orange.

Poppe and Gyles (1988) compared the effectiveness of a number of procedures designed to eliminate the virulence plasmids of salmonella. They concluded that the plasmids were more readily eliminated by incubation at 45.5°C than by chemical curing. None was cured by acridine orange, novobiocin and SDS although EB was proved to be of some value.

Verma (1988) suggested that EB efficiently eliminated plasmids in different salmonellae.

Anjanappa *et al.* (1993) reported the elimination of R plasmids in *S. gallinarum* by culturing at 45°C for 50 days but curing with chemical agents, EB and novobiocin occurred infrequently.

Poppe *et al.* (1993) observed curing of plasmids by incubation of the plasmid containing *S. enteritidis* strains at 45°C for two to seven days.

2.9 Pathogenicity studies

Jones *et al.* (1982) reported that strains of *S. typhimurium* cured of 60 Mdal plasmid were less invasive and adhesive. One cured derivative was avirulent and the other one was 10^3 to 10^5 times less virulent for orally infected mice as compared with their respective parental strains.

Terakado *et al.* (1983) showed that *Salmonella dublin* organisms cured of 50 Mdal plasmid were 100-1000 fold less virulent to mice when given intraperitoneally than were wild strains.

Helmuth *et al.* (1985) performed the virulence studies with 39 representative strains after oral infection of mice. The LD_{50} values obtained for plasmid positive strains of *S. typhimurium*, *S. enteritidis* and *S. dublin* were upto 10^6 fold lower than the values obtained for the plasmid free strains of the same serotype.

Nakamura *et al.* (1985) observed that a 36 Mdal plasmid in *S. enteritidis* was associated with virulence in orally and subcutaneously infected mice but not the wild type strain harbouring the plasmid.

Barrow *et al.* (1987) assessed the contribution of the plasmids to virulence of *S. gallinarum* by oral and intramuscular inoculation of newly hatched and two week old chickens. Elimination of the large plasmid resulted in a marked loss of virulence and reintroduction of the plasmid restored complete virulence.

Poppe and Gyles (1987) tested the virulence of several salmonella serotypes using BalbC mice and day old chicks by oral administration and intraperitoneal (i/p) inoculation and proved that only i/p inoculated chicks were useful in demonstrating differences among the isolates. Barrow and Lovell (1988) showed that following oral administration of newly hatched chicken with the parent strain of *S. pullorum* having 85 kb large plasmid produced a high (71%) mortality but not the derivatives that lack this plasmid.

Williamson *et al.* (1988) stated that the cured derivatives of *S. dublin* and *S. typhimurium* showed reduced virulence of 10^4-10^5 and 10^2 respectively after oral infection of mice.

2.10 REAC of various Gram-negative bacteria

REA of bacterial DNA has been successfully used for the accurate subspecific identification and/or intragenenic characterisation of a number of bacteria.

Genomic DNA fingerprinting provided the first definite test for closely related markers of the genus Bacillus. A reproducible pattern of bands were visible after electrophoresis of restriction enzyme cleaved genomic DNA for *E. coli*, *B. subtilis* and *Rhizobium* species (Corfield *et al.*, 1987).

Wasteson *et al.* (1992) performed REA of total DNA with HaeIII to distinguish between the different *E. coli* strains isolated from pigs with edema disease.

Digestion of chromosomal DNA from 18 field strains of *S. enteritidis* and 12 *S. typhimurium* with PstI and subsequent separation of fragments by pulse field electrophoresis in agarose gel yielded three and nine chromosomal types in *S. enteritidis* and *S. typhimurium* respectively (Rychlik *et al.*, 1993).

Smart *et al.* (1993) used RE fragmentation analysis technique for epidemiological investigation of the Enzootic Glassers disease caused by *H. parasuis* on a farm where all the strains were biochemically similar and many strains are biochemically untypable.

Gardner *et al.* (1994) assessed the differences in four phenotypes of *Pasteurella multocida* by REA of whole cell DNA with SmaI.

Blackall *et al.* (1995) concluded that REA and ribotyping were superior to biotyping methods for the investigation of fowl cholera outbreaks. REA performed with HpaII established seven groups from 22 isolates.

Miflin *et al.* (1995) performed REA of *H. paragallinarum* with three enzymes HindIII, HpaII and SspI. All isolates gave identical REA profiles with all three enzymes.

On analysis of isolates *P. multocida* belonging to three antigenic groups, Wilson *et al.* (1995) observed identical NciI fingerprint profiles. Differentiation of field isolates was achieved by digestion of DNA with HpaII.

Delong *et al.* (1996) examined REA pattern of DNA which were digested with cfoI indicated clear differences correlating with species and biogroups in camphylobacter species.

Hartmann and West (1997) reported that digestion of genomic DNA from multiresistant *S. anatum* isolated from horses with three different enzymes SfiI, SpeI and XbaI followed by pulse field gel electrophoresis revealed a highly conserved restriction endonuclease digestion pattern.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Identification of salmonella

Four serotypes of salmonella isolated from diseased birds and one serotype isolated from eggs which were available in the Department of Microbiology were used for this study.

Smooth non lactose fermenting colonies on Mac Conkey agar (MCA, Hi-media) and Brilliant Green agar (BGA, Hi-media) were inoculated onto Triple Sugar Iron (TSI, Hi-Media) agar by stabbing the butt and streaking the slant. The organisms which showed the typical characteristic growth on TSI agar were identified by morphological characters including staining reaction and biochemical tests. The following biochemical tests were performed viz., catalase, oxidase, oxidative/fermentative test, indole production, nitrate reduction, methyl red test, Voges Proskauer test, urease, citrate utilisation, gelatin liquefaction, growth in KCN medium, phenyl alanine deaminase, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, esculin hydrolysis, O-nitrophenyl-ß-D-galactopyranoside (ONPG) and sugar fermentation tests.

3.2 Serotyping

Serotyping was carried out using the polyvalent and factor sera available in the Department of Microbiology by plate agglutination test.

3.3 Antibiogram

3.3.1. Agar diffusion method

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

3.3.2 Media

Mueller Hinton agar (MHA) was used.

3.3.3 Antibiotic discs (Hi-Media)

The following antibiotic/chemotherapeutic discs were used with their potency in micrograms (µg) per disc.

1. Ampicillin (A) - 10 µg
2. Cephalothin (Ch) - 30 µg
3. Chloramphenicol (C) - 30 µg
4. Ciprofloxacin (Cf) - 5 µg
5. Cloxacillin (Cx) - 5 µg
6. Cotrimazine (Cm) - 25 µg
7. Doxycycline (D) - 30 µg
8. Enrofloxacin (Ex) - 10 µg
9. Furazolidone (F) - 50 µg
10. Gentamicin (G) - 10 µg
11. Kanamicin (K) - 30 µg
12. Nalidixicacid (Na) - 30 µg

13. Neomycin (N) - 30 µg
14. Nitrofurantoin (Nf) - 3000 µg
15. Oxytetracycline (O) - 30 µg
16. Rifampicin (R) - 5 µg
17. Streptomycin (S) - 10 µg
18. Sulfadiazine (Sz) - 300 µg
19. Trimethoprim (Tr) - 5 µg
20. Triple sulpha (S_3) - 250 µg

3.4 Plasmid profile of avian salmonellae

3.4.1 Buffers and Reagents

The following buffers and reagents were prepared and used as per Sambrook *et al.* (1989) with slight modifications.

3.4.1.1 Luria Bertani medium (LB) (Hi-media)

Bacto-tryptone	-	10 g
Bacto yeast extract	-	5 g
Sodium chloride	-	10 g
Distilled water	-	1 L

pH was adjusted to 7.4 and autoclaved at 121°C at 15 lbs pressure for 15 min.

3.4.1.2 Tris- EDTA glucose (TEG) buffer (pH 8.0)

Glucose	-	50 mM
Tris-cl (pH 8.0)	-	25 mM
EDTA (pH 8.0)	-	10 mM
Distilled water to make upto-		100 ml

Autoclaved at 10 lbs pressure for 20 minutes and stored at 4°C.

3.4.1.3 Lysozyme (Merck)

3.4.1.4 SDS- NaOH solution

Sodium hydroxide	-	0.2 N
Sodium dodecyl sulphate	-	1.0 g
Distilled water	-	100 ml

SDS-NaOH solution was made up from stock solutions of 10 per cent SDS and 1N NaOH.

3.4.1.5 Sodium acetate solution

3 M Sodium acetate	- 40.81 g
Distilled water to make up to	- 100 ml

pH was adjusted to 4.8 with glacial acetic acid. Autoclaved at 121°C for 15 min at 15 lbs pressure.

3.4.1.6 Distilled ethanol

3.4.1.7 Phenol chloroform mixture (24:25 ratio)

"Phenol" means phenol equilibrated with buffer and containing 0.1 per cent hydroxyquinolone and 0.2 per cent mercaptoethanol.

"Chloroform" means a 24:1(V/V) mixture of chloroform isoamyl alcohol mixture.

3.4.1.8 Chloroform isoamyl alcohol mixture

A mixture of chloroform and isoamyl alcohol (24:1 V/V) was used to remove proteins from preparation of nucleic acid. The chloroform denatures protein while isoamyl alcohol reduces foaming during the extraction and facilitates the separation of the aqueous from organic phase. The mixture is stable and was stored in closed containers at room temperature.

3.4.1.9 Tris EDTA (TE) buffer (pH 8.0)

Tris- Cl (pH 8.0)	- 10 mM
EDTA (pH 8.0)	- 1 mM
Distilled water	- 100 ml

Autoclaved at 121°C for 15 min at 15 lbs pressure.

3.4.1.10 RNase that is free from DNase

Pancreatic RNase (Sigma) was dissolved at a concentration of 10 mg/ml in TE buffer. The solution was heated to 100°C for 15 min and allowed to cool at room temperature slowly. It was then dispersed into 1 ml aliquots and stored at -20°C.

3.4.1.11 Tris Borate Electrophoresis (TBE) buffer stock solution 5x

Tris base	- 54.0g
Boric acid	- 27.5 g
EDTA (0.5 M)	- 20.0 ml
Distilled water	- 980 ml

Autoclaved at 121°C for 15 min at 15 lbs pressure. The stock solution was diluted to 1x before use.

3.4.1.12 Gel loading buffer

Bromophenol blue	- 0.25 per cent
Xylene cyanol	- 0.25 per cent
Glycerol	- 30 per cent

3.4.1.13 Agarose (Sigma)

3.4.1.14 Ethidium bromide (Sigma) (EB)

Ethidium bromide at a concentration of 0.5 µg/ml of sterile distilled water was prepared and used.

3.4.2 Plasmid analysis of salmonellae

The plasmid DNA isolation was done from five cultures of avian salmonellae by alkaline lysis technique of Birnboim and Doly (1979) with the modification of Sambrook *et al.*, (1989).

The three basic steps involved in the plasmid DNA preparations are.

1. Growth of the bacteria
2. Harvesting and lysis of the bacteria
3. Isolation and precipitation of plasmid DNA.

3.4.2.1 Growth of the bacteria

A single bacterial colony from a pure culture of salmonella colonies was inoculated into 2 ml of LB medium.

After 6 h, the 2 ml of culture was transferred into 10 ml of LB broth and incubated overnight at 37°C with frequent shaking.

3.4.2.2 Harvesting and lysis of bacteria

Bacterial culture (1.5 ml) was transferred into an eppendorf tube and the cells were harvested by centrifuging at 6000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was allowed to dry.

The bacterial pellet was resuspended in 100 µl of ice cold TEG solution containing lysozyme at the concentration of 2 mg/ml by gentle mixing. It was kept at room temperature for 15 min.

Later 200 µl of freshly prepared SDS-NaoH solution was added and the contents were mixed by inverting the tubes 4-5 times. The tubes were placed over ice for 15 min.

Then 150 µl of ice cold solution of 3 M sodium acetate solution was added. The tubes were inverted gently for thorough mixing and kept on ice for 15 min.

The tubes were then centrifuged at 15,000 rpm for 10 min in microfuge. The supernatent was transferred to a fresh tube.

3.4.2.3 Plasmid DNA purification

3.4.2.3.1 Extraction with phenol chloroform

To the measured volume of the supernatant solution, equal volume of phenol chloroform was added and mixed in a polypropylene tube with a plastic cap. The contents were mixed till an emulsion was formed. Then centrifuged at 3000 rpm for 10 min at room temperature. When the organic and aqueous phases

are not well separated, centrifugation was further continued and the aqueous phase was transferred to a fresh tube.

3.4.2.3.2 Extraction with chloroform Isoamyl alcohol

Equal volume of chloroform isoamyl alcohol mixture was added to plasmid DNA solution and mixed thoroughly. It was centrifuged at 3000 rpm for 10 min at room temperature. After a distinct separation of aqueous and organic phases the aqueous phase was collected and transferred to a fresh tube.

3.4.2.4. Isolation and precipitation of DNA with ethanol

To the aqueous phase double volume of ethanol was added and the mixture was kept at -20°C overnight.

The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 20 min in the microfuge.

The pellet was rinsed in 1 ml of 70% ethanol to remove any solute that may be trapped in the precipitate. The supernatant was discarded and the pellet was allowed to dry.

The pellet was dissolved in 50 µl of TE buffer. To the DNA solution, added 5 µl of RNAse and incubated at 37°C for 30 min.

3.4.2.5 Measurement of concentration of plasmid DNA

The purity and concentration of plasmid DNA were assessed by spectrophotometry. The ratio between the readings at 260 nm and 280 nm (OD 260/UD 280) provides an estimate for purity of the nucleic acid. It should be 1.8.

The UV spectrophotometer readings after standardisation with TE buffer as blank was used for taking the OD readings of the prepared plasmid DNA samples from salmonella at 260 nm and 280 nm wavelengths. The concentration was calculated using the formula OD 1 at 260 nm = 50 μg/ml (for double stranded DNA).

3.4.2.6 Agarose gel electrophoresis (AGE)

This was carried out as per the modification of Meyers *et al.* (1976).

Agarose (120 mg) was dissolved in 15 ml of TBE buffer (0.8%) by heating and then cooled to 50°C and the intercalating dye ethidium bromide was added to a final concentration of 0.5 μg/ml. The clean, dry gel platform edges were sealed with adhesive tape and the comb was kept in position before pouring the agarose solution into the glass plate for setting. While doing so, a gap of 0.5-1.0 mm has been provided between the glass plate and the comb tip so as to form a seal of agarose within the well formed beneath the comb tip. Thirty to 45 min were allowed to lapse for the agarose solution to set into a gel. After removing the tape and the comb, the platform with gel was placed within the tank filled with

TBE (1x) buffer and some more TBE buffer was added if necessary until the agarose gel got completely submerged. To 20 µl of DNA samples 5 µl of loading buffer was added and loaded into each slot of the prepared gel.

Electrophoresis was carried out at 35 mA for 2 h or till the loading dye reached 3/4 of the gel. The gel was examined in Alpha Imager (Alpha Innotech Corporation USA).

3.4.2.7 Photography of plasmid DNA

The gel was viewed and photographed in the Alpha Imager.

3.4.2.8 Estimation of molecular size of plasmid DNA

The molecular size of plasmid DNA was estimated by drawing a linear graph in comparison with the molecular size of the plasmids of *E. coli* V517. (Macrina *et al.*, 1978).

A standard curve was drawn by plotting the $\log_{10}$ kb values of the plasmid DNA of the marker strain V517 on Y axis and distance migrated on X axis. The values of the distance migrated by the unknown plasmids were interpolated with the standard curve to arrive at the molecular weight of plasmid DNA.

3.5 Restriction enzyme analysis of plasmid DNA.

3.5.1 Restriction Enzymes(RE)

3.5.1.1. EcoRI (Genei)

10x assay buffer B

3.5.1.2 HindIII (Genei)

10x assay buffer C

3.5.2 Digestion

The five plasmid DNA isolated were digested with the restriction endonuclease enzymes EcoRI and HindIII, according to the method described by Maniatis *et al.* (1982) DNA (10-20 µg), RE assay buffer (2 x concentration) and RE (2 units/µg) were added in eppendorf tubes containing distilled water so that the total reaction volume of each tube would be 20 µl. The digestion mixture was incubated at 37°C for 1 hour. The restriction enzyme activity was inactivated by heating at 65°C for 20 min before electrophoresis.

3.5.3 Agarose gel electrophoresis

It was performed using the procedure described under plasmid DNA electrophoresis.

3.5.4 Photography

The gel was viewed and photographed in the Alpha Imager.

3.5.5 Molecular size estimation

The DNA HindIII digest was run along with the sample DNA and the standard graph was plotted from the mobility of known fragments of the marker for the molecular weight determination of enzyme digested fragments of plasmid DNA.

3.6 Curing of Plasmids

The method of Carlton and Brown (1981) was applied for curing of plasmids.

3.6.1 Curing with ethidium bromide

Ethidium bromide at a concentration of 100 µg/ml was used. From a 6 h old culture, 20 µl was inoculated into 5 ml of LB broth containing 100 µg/ml of EB.

The cultures were incubated at 37°C for seven days but subcultured on MCA plates (Master plate) every 24 hours to obtain single well isolated colonies.

The master plate was replica plated to another MCA plate containing one of the antibiotics(selective medium) to which the host strain was resistant and incubated at 37°C for 24h. A colony was considered to be cured when it failed to

grow on antibiotic containing MCA (selective medium) but grew on antibiotic free MCA.

3.6.2 Curing with Acridine orange (AO)

The curing with AO was carried out by treating the organisms at the concentration of 100 µg/ml of AO in 5 ml of LB broth at 37°C for 24h. The curing effect was examined by replica plating as done previously.

3.6.3 Curing with sodium dodecyl sulphate

The curing with SDS was carried out by culturing the organisms in 5 ml of LB broth containing 10 per cent SDS (100 mg/ml). The cultures were incubated at 37°C for three days. The curing effect was examined every 24 hours by replica plating.

3.6.4 Curing at elevated temperature

Salmonella isolates were incubated in 5 ml of LB broth at 45°C for 14 days. The cultures were transferred every 24h to fresh LB broth for the next 14 days and the colonies were examined by replica plating on MCA containing antibiotic at an interval of five days.

The cured strains obtained by the above methods were verified for the loss of plasmid (s) and resistance pattern by plasmid and antibiogram studies.

3.7 Pathogenicity studies

Forty eight day old chicks were procured from university poultry farm, Mannuthy. The chicks were divided into sixteen groups, each containing three birds. Birds were fed with chick mash and watered ad libitum. The pathogenicity of wild and cured isolates were studied as per the procedure described by Poppe and Gyles (1987).

Overnight cultures of bacteria in LB broth were diluted 1:100 in fresh LB broth and incubated with shaking for 4 h at 37 °C to a density of approximately 2×10^9 colony forming units/ml. The chicks were challenged orally (0.2 ml) and intraperitoneally (0.1 ml) with wild type isolates and plasmid cured isolates. The chicks were observed for 14 days. Reisolation of the organism from liver, spleen and caecal tonsils of ailing/dead chicks was attempted.

3.8 Restriction enzyme analysis of chromosomal DNA (REAC)

3.8.1 Buffers and Reagents

3.8.1.1. 0.1 M PBS

Sodium Chloride	- 8 g
Potassium chloride	- 0.2g
Disodium hydrogen phosphate	- 1. 608 g
Potassium dihydrogen phosphate	- 0.2g
Distilled water	- 1000 ml

3.8.1.2. TE buffer pH 8.0 (3.4.1.9)

3.8.1.3 Lysozyme (Merck)

Fifteen milligram of lysozyme was dissolved in 5 ml of distilled water and stored at -20°C .

3.8.1.4 Proteinase K (Merck)

Ten milligram of proteinase K was dissolved in 1 ml of distilled water. It was initially preincubated at 37°C for 2 hours and stored at -20°C.

3.8.1.5 SDS solution (10 per cent)

3.8.1.6 Phenol chloroform (3.4.1.7)

3.8.1.7 Distilled ethanol

3.8.1.8 Gel loading buffer (3.4.1.12)

3.8.1.9 Agarose

3.8.1.10 Restriction enzymes (3.5.1)

3.8.1.11 Marker

Lamda DNA/HindIII digest (Genei) concentration 300 µg/ml.

3.8.2 DNA isolation from salmonella cultures.

Total DNA was isolated and digested with the restriction endonuclease enzymes according to the method described by Maniatis *et al.* (1982).

The procedure for total DNA preparation from a culture of bacterial cells could be divided into four stages.

1. Pure culture of bacteria was grown and then harvested
2. The Cells were broken open to release their contents
3. The cell extract was treated to remove all components except the DNA.
4. The resulting DNA solution was concentrated.

3.8.2.1 Growing and harvesting of bacterial culture

Bacteria were grown in 10ml of LB medium overnight with frequent shaking for aeration. The growth of the culture was monitored by reading the optical density (OD) at 600nm at which wavelength one OD unit corresponds to about 0.8×10^9 cells/ml.

In order to prepare a cell extract, the bacteria must be obtained in as small a volume as possible. Harvesting was therefore performed by spinning the culture at 6000 rpm for 20 min in a centrifuge.

The pellet obtained was washed two times with PBS pH 7.2 0.1 M.

The pellet was then resuspended in one ml of TE buffer.

3.8.2.2 Preparation of cell extract

To the pellet prepared, 100 µl of lysozyme solution was added and incubated in a 37°C water bath for 15 min.

Later, 100 µl of SDS solution and 100 µl of proteinase K solution were added and incubated at 50°C waterbath overnight.

3.8.2.3 purification of DNA from the cell extract

Equal volume of phenol chloroform (25:24 ratio) was added to the cell extract and mixed gently for about 5-10 min for deproteinization.

It was spinned at 2000 rpm for 15-30 min in a cooling centrifuge and the upper aqueous phase containing DNA was transferred to another tube. Care was taken not to disturb the precipitated protein molecules which were left as a white coagulated mass at the interphase between the aqueous and organic layers. This extraction step was repeated two more times to purify the nucleic acids.

3.8.2.4 concentration of DNA

The ice cold ethanol was layered on top of the DNA sample causing molecules to precipitate at the interphase. A glass rod was pushed through the ethanol into the DNA solution and when the rod was removed,the DNA adhered onto it was pulled out in the form of a long fibre. When the concentration of DNA was less, it was recovered by centrifugation.

3.8.2.5 Measurement of concentration of DNA

Both purity and concentration were determined by spectrophotometry. An absorbance of 1.0 at 260 nm wavelength corresponds to 50µg of double stranded

DNA per ml. The ratio between the readings at 260nm and 280nm (OD 260/OD 280) should be 1.8 which indicates the purity of DNA. DNA samples having ratio of less than 1.8 were subjected to the procedure from the addition of proteinase K step once again to obtain sufficiently pure DNA.

3.8.3 Restriction digestion

DNA (10-20 μg), RE assay buffer (2x concentration) and RE were taken on microfuge tubes containing distilled water so that the total reaction volume in each tube would be 20 μl. The digestion mixture was incubated at 37°C overnight. Restriction enzyme activity was inactivated by heating at 65°C for 20 min before electrophoresis.

3.8.3.1 Analysis of the result of restriction endonuclease cleavage

Separation of fragments by gel electrophoresis was performed as described under plasmid DNA electrophoresis but in 1 per cent agarose gel. 300 mg of agarose was disslved in 30 ml of TBE buffer.

Each sample was loaded in the gel and electrophoresis was carried out at 50 V for six hours.

3.8.3.2 Photography

The gel was viewed and photographed in the Alpha Imager.

3.8.3.3 Estimation of molecular size of digested fragments

The digested total DNA of salmonella culture were electrophoresed together with Lamda DNA HindIII digest as molecular weight standard.

Results

4. RESULTS

4.1 Identification

Five avian strains salmonellae available in the Department of Microbiology were used in this study and they were further identified.

The cultures were streaked onto MCA and BGA plates. pale, nonlactose fermenting smooth colonies of 1-2 mm in diameter on MCA and pink colonies of 1-2 mm in diameter with pink zone surrounding them on BGA plates were obtained. The organisms showed the typical characteristic growth on TSI agar i.e. acid butt (yellow); alkaline slant (pink) with hydrogen sulphide production (blackening).

The results of study of morphological characters – staining and biochemical reactions including sugar fermentation tests are furnished in Table 1, 2a and 2b.

All isolates were Gram negative bacilli. All were motile except *S. gallinarum*, catalase positive, oxidase negative, grew aerobically and anaerobically, fermented glucose and were non hemolytic on blood agar.

In the secondary tests, all were indole negative, reduced nitrate, methyl red positive, Voges-Proskauer negative, urease negative, utilised citrate, gelatin liquefaction negative, growth in KCN medium negative, phenyl alanine deaminase negative, lysine decorboxylase positive, esculin hydrolyis negative and ONPG

negative. The three paratyphoid organisms (SE, ST and SB) showed positive reactions in arginine dihydrolase and ornithine decorboxylase and the nonmotile isolates (SG1 and SG2) showed negative reactions. All the isolates utilised arabinose, dulcitol, D glucose, maltose, mannitol, D mannose, trehalose and D-xylose but not D-adonitol, cellobiose, lactose, raffinose and sucrose. But nonmotile isolates did not utilise melibiose, rhamnose and sorbitol.

4.2 Serotyping

Salmonella enteritidis (9,12:g,m:-), *S. branderup* (6,7:e,h:e,n,z_{15}) and *S. typhimurium* (1,4,12:i:1,2) were serotyped at the National Escherichia and Salmonella Centre, Kasauli H.P. *S. gallinarum* (SG1 and SG2) SE, SB and ST were serotyped using the polyvalent and factor sera available in the Department of Microbiology and the results of the reactions are furnished in Table 3.

4.3 Antibiogram

The results of antibiogram of avian salmonellae to various antibiotics/ chemotherapeutic agents is presented in Table 4.

All serotypes were resistant to cloxacillin and rifampicin but sensitive to ampicillin, ciprofloxacin, cotrimazine, enrofloxacin, kanamicin, nalidixic acid, neomycin, nitrofurantoin and streptomycin. SB was quadruple resistant, SG1 and SG2 were quintuple resistant, SE was sextuple resistant, ST was septuple resistant. Using 20 antibiotics/chemotherapeutic agents four different resistance patterns were observed within five salmonellae.

4.4 Plasmid profile

The plasmid profile of avian salmonellae was analysed by agarose gel electrophoresis (Fig.1). The number of plasmids present in these strains varied from zero to three and the size of the plasmids ranged from 1.75 kb to 48.32 kb (Table 5).

SE and SB were without any plasmids while in the other three, three different patterns of plasmid profiles were observed. Both SG1 and SG2 had low molecular weight plasmids of 2.6 and 1.75 kb and 2.6 and 1.8 kb respectively. But SG1 had one additional plasmid of 48.32 kb in size. ST had two plasmids of sizes 13.62 kb and 4.2 kb.

4.5 Restriction enzyme analysis of plasmid DNA

The plasmid DNA preparation from three of the isolates was digested with EcoRI and HindIII separately and the fragments generated were analysed by agarose gel electrophoresis (Fig.2).

EcoRI cleaved the large plasmid of 48.32 kb present in SG1 into three fragments of sizes 18.84, 16.25 and 13.23 kb. Likewise HindIII cleavage resulted in three fragments of sizes 21.50, 17.02 and 9.80 kb yielding a total of 48.32 kb. Among the common plasmids present in SG1 and SG2, 2.6 kb plasmid remained uncleaved and the approximately 1.8 kb plasmid was cleaved by both enzymes but the fragments of which could not be detected on the gel after electrophoresis. In ST, EcoRI digestion of 13.62 kb DNA yielded two fragments of 8.7 and 4.92 kb

and for the other plasmid of 4.2 kb, one fragment of 2.7 kb was only detected. In the same isolate, HindIII cleavage of 13.62 kb plasmid DNA resulted in three fragments of sizes 6.43, 4.87 and 2.32 kb and for the other plasmid of 4.2 kb, only a fragment of 2.96 kb was detected on the gel.

Three different restriction patterns were observed in each of the isolate after digestion with EcoRI and HindIII enzymes separately. The correlation between plasmid profile and restriction enzyme analysis of plasmid DNA is furnished in Table 6.

4.6 Curing of plasmids and antibiogram of cured isolates

The curing effect of elevated temperature and chemicals to three isolates of salmonellae are presented in Table 7.

4.6.1 Curing with EB

Ethidium bromide eliminated the small and large plasmids present in SG1, SG2 and ST within 48 hours of incubation. The loss of which did not result in loss of any resistance character in both SG1 and SG2. But the loss of plasmids in ST resulted in loss of resistance character to four antibiotics, viz., chloramphenicol, sulfadiazine, triple sulphas and cotrimazine.

4.6.2 Curing with AO

The plasmids were not lost from anyone of the isolates after 24 hours of incubation.

4.6.3 Curing with SDS

The plasmids present in anyone of the isolates were not eliminated even after incubation for three days.

4.6.4 Curing at elevated temperature

The plasmids present in three of the isolates were eliminated only after two weeks of incubation. The isolates undergone curing were subjected to plasmid profile studies and antimicrobial sensitivity testing to find out the loss of resistance and plasmids.

4.7 Pathogenicity studies

The results of pathogenicity studies on day old chicks inoculated with wild and cured strains of SG1, SG2 and ST and wild isolates of SE and SB orally as well as intraperitoneally are presented in Table 8.

The birds were dull, depressed, anorectic, stood with lowered heads and closed eyes and profuse watery diarrhoea before death. The lesions observed were severe congestion on all organs, unabsorbed yolk, severe haemorrhagic enteritis.

Day old chicks challenged with wild isolates of SE and SB caused death of chicks on first or second day. Both wild and cured isolates of SG1 and SG2 did not produce any clinical symptom or death of chicks and the birds remained apparently normal throughout the observation period of 14 days. Oral and intraperitoneal inoculation of wild isolate of ST was lethal to all the birds. Oral

administration of cured isolate of ST resulted in death of two birds but the chicks inoculated intrperitoneally remained normal throughout the observation period.

The bacteria were reisolated from spleen, liver, caecal tonsils and from unabsorbed yolk.

4.8 Restriction enzyme analysis of chromosomal DNA

The chromosomal DNA isolated from all the five avian salmonellae were subjected to digestion trials with EcoRI and HindIII and the fragments were analysed after AGE (Fig.3). Indistinct separation of bands only were seen in all the isolates after digestion with EcoRI and HindIII.

Table 1. Primary identification tests of avian salmonellae

Tests	Isolates				
	SE	SG1	SG2	ST	SB
Grams reaction	-	-	-	-	-
Shape	bacilli	bacilli	bacilli	bacilli	bacilli
Presence of capsule	-	-	-	-	-
Motility	+	-	-	+	+
Growth aerobically	+	+	+	+	+
Growth anaerobically	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Oxidative/fermentation (O/F)	F	F	F	F	F
Hemolysis on blood agar	-	-	-	-	-

Table 2 a. Second stage biochemical tests of avian salmonellae

Tests	Isolates				
	SE	SG1	SG2	ST	SB
Indole	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Methyl red test	+	+	+	+	+
Voges Proskauer test	-	-	-	-	-
H_2S production on TSI	+	+	+	+	+
Urease	-	-	-	-	-
Citrate utilisation	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-
Growth in KCN medium	-	-	-	-	-
Phenyl alanine deaminase	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+
Arginine dihydrolase	+	-	-	+	+
Ornithine decorboxylase	+	-	-	+	+
Esculin hydrolysis	-	-	-	-	-
ONPG	-	-	-	-	-

Table 2 b. Sugar fermentation reactions of avian salmonellae

Tests	Isolates				
	SE	SG1	SG2	ST	SB
D-Adonitol	-	-	-	-	-
L-Arabinose	+	+	+	+	+
Cellobiose	-	-	-	-	-
Dulcitol	+	+	+	+	+
D-glucose	+	+	+	+	+
Lactose	-	-	-	-	-
Maltose	+	+	+	+	+
D-mannitol	+	+	+	+	+
D-mannose	+	+	+	+	+
Melibiose	+	-	-	+	+
Raffinose	-	-	-	-	-
L-Rhamnose	+	-	-	+	+
Salicin	-	-	-	-	-
D-sorbitol	+	-	-	+	+
Sucrose	-	-	-	-	-
Trehalose	+	+	+	+	+
D-xylose	+	+	+	+	+

Table 3. Serotyping of avian salmonellae

Isolates	Agglutination										
	Polyvalent O antisera	Factor sera									
		2	4	7	8	9	3,10	1,3,19	11	13	12	6
SE	+	-	-	-	-	+	-	-	-	-	+	-
SG1	+	-	-	-	-	+	-	-	-	-	+	-
SG2	+	-	-	-	-	+	-	-	-	-	+	-
ST	+	-	+	-	-	-	-	-	-	-	+	-
SB	+	-	-	+	-	-	-	-	-	-	-	+

Table 4. Antibiogram of avian salmonellae

	A	Ch	C	Cf	Cx	Cm	D	Ex	F	G	K	Na	N	Nf	O	R	S	Sz	Tr	S3
SE	S	R	S	S	R	S	S	S	S	R	S	S	S	S	S	R	S	R	S	R
SG1	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	R	S	R	S	R
SG2	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	R	S	R	S	R
ST	S	R	R	S	R	S	S	S	R	S	S	S	S	S	S	R	S	R	S	R
SB	S	S	S	S	R	S	R	S	S	S	S	S	S	S	R	R	S	S	S	S

Table 5. Antibiogram pattern and plasmid profile of avian salmonellae

Isolates	Antibiogram	No. of antibiotics Resistant	Plasmid profile	Molecular size of (kb) plasmids
SE	Ch C x GR Sz S3	6	-	-
SG1	Ch C x R Sz S3	5	3	48.32, 2.6, 1.75
SG2	Ch C x R Sz S3	5	2	2.6, 1.8
ST	Ch C C x FR Sz S3	7	2	13.62, 4.2
SB	C x DOR	4	-	-

Table 6. Restriction enzyme analysis of plasmid DNA of avian salmonellae

Isolates	Molecular size of plasmid (in kb)	Enzyme used	Molecular size of fragments (in kb)
SGI	48.32, 2.6, 1.75	EcoR1	18.84, 16.25, 13.23, 2.6
		Hind III	21.50, 17.02, 9.80, 2.6
SG2	2.6, 1.8	EcoR1	2.6
		Hind III	2.6
ST	13.62, 4.2	EcoR1	8.7, 4.92, 2.7
		Hind III	6.43, 4.87, 2.96, 2.32

Table 7. Curing of plasmids of avian salmonellae by chemical and physical methods

Isolate	Curing agent	Resistance pattern	Time taken (in days)	Loss of resistance pattern
SGI	EB	Ch C x R Sz S3	2	-
	SDS		-	-
	AO		-	-
	Elevated temperature		14	-
SG2	EB	ChC x R Sz S3	2	-
	SDS		-	-
	AO		-	-
	Elevated temperature		-	-
ST	EB	ChCC x FR Sz S3	2	CCmSz S3
	SDS		-	-
	AO		-	-
	Elevated temperature		14	CCm Sz S3

Table 8. Pathogenicity studies of avian salmonellae in day old chicks

Isolate	No. of plasmids present	No. of plasmids present after curing	No. of birds used	Quantity given (in ml)	Route	Time of death	No. of birds died
SE	-	-	3	0.2	Oral	48, 72	2, 1
(Wild)			3	0.1	i/p	24	3
SGI	3	-	3	0.2	Oral	-	-
(Wild)			3	0.1	i/p	-	-
							-
SG1	3	0	3	0.2	Oral	-	-
(Cured)			3	0.1	i/p	-	-
SG2	2	-	3	0.2	Oral	-	-
(Wild)			3	0.1	i/p	-	-
SG2	2	0	3	0.2	Oral	-	-
(Cured)			3	0.1	i/p	-	-
ST	2	-	3	0.2	Oral	48, 72	2, 1
(Wild)			3	0.1	i/p	24	3
ST	2	0	3	0.2	Oral	48	2
(Cured)			3	0.1	i/p	-	-
SB	-	-	3	0.2	Oral	24, 48	1, 2
(Wild)				0.1	i/p	24, 48	2, 1

Fig.1 Plasmid profile of avian salmonellae

Lane 1 : E. coli V517 with 8 plasmids of standard size

Lane 2 : SE with no plasmids

Lane 3 : SG1 with 3 plasmids of 48.32 kb, 2.6 kb and 1.75 kb

Lane 4 : SG2 with 2 plasmids of 2.6 kb and 1.8 kb

Lane 5 : ST with 2 plasmids of 13.62 kb and 4.2 kb

Lane 6 : SB with no plasmids

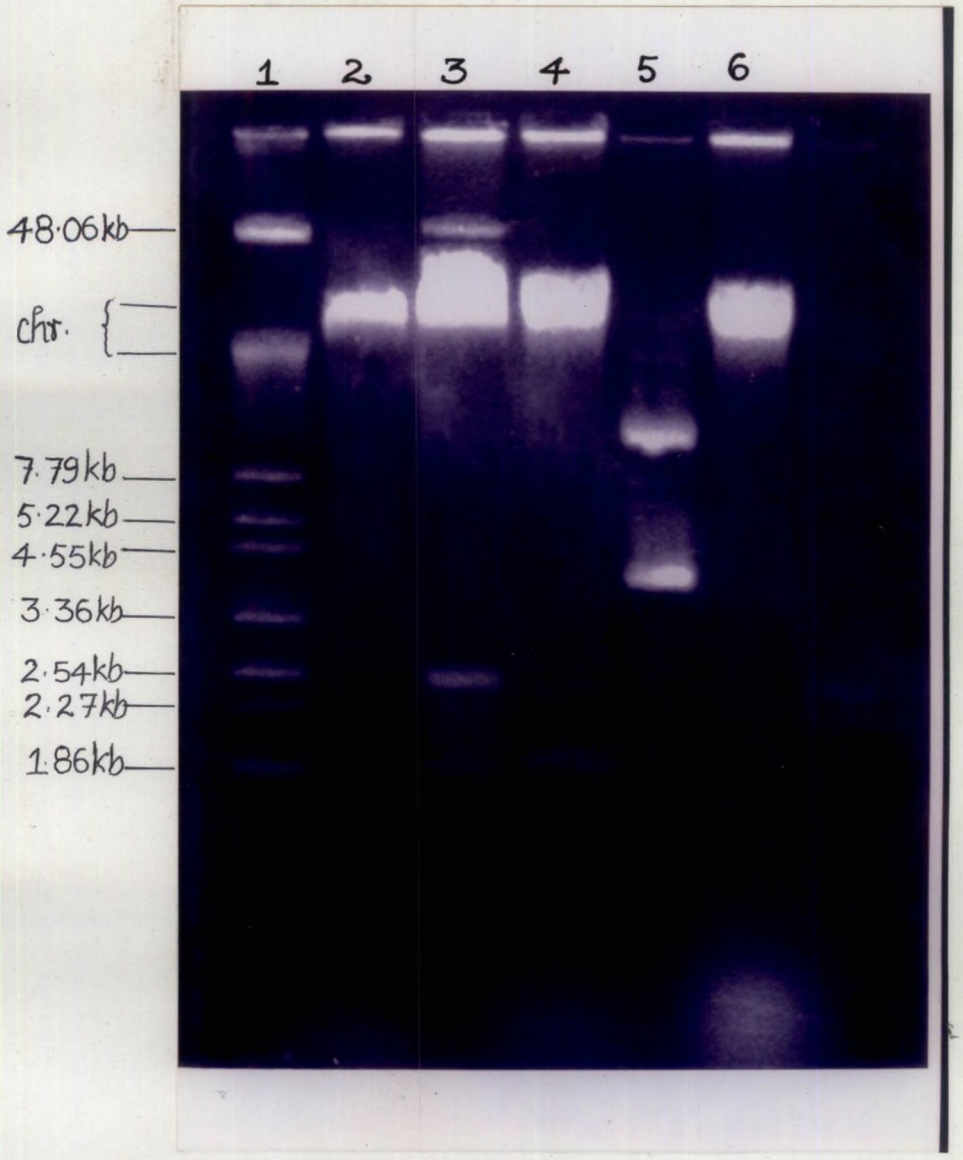

1
2
3
4
5
6
48·06kb
chr.
7·79kb
5·22kb
4·55kb
3·36kb
2·54kb
2·27kb
1·86kb

Fig.2 Restriction enzyme analysis of plasmid DNA

Lane 1 : Uncleaved 2.6 kb plasmid after EcoRI digestion (SG2)

Lane 2 : EcoRI digest of 48.32 kb plasmid showing 3 fragments of 18.84 kb, 16.25 kb and 13.23 kb and an uncleaved plasmid of 2.6 kb (SG1)

Lane 3 : EcoRI digest of 13.62 kb and 4.2 kb plasmids showing 3 fragments of 8.7 kb, 4.92 kb and 2.7 kb (ST)

Lane 4 : Uncleaved 2.6 kb plasmid after HindIII digestion (SG2)

Lane 5 : HindIII digest of 48.32 kb plasmid showing 3 fragments of 21.50 kb, 17.02 kb and 9.80 kb and an uncleaved plasmid of 2.6 kb (SG1)

Lane 6 : HindIII digest of 13.62 kb and 4.2 kb plasmids showing 4 fragments of 6.43 kb, 4.87 kb, 2.96 kb and 2.32 kb (ST)

Lane 7 : λDNA HindIII digest with 6 fragments of standard size

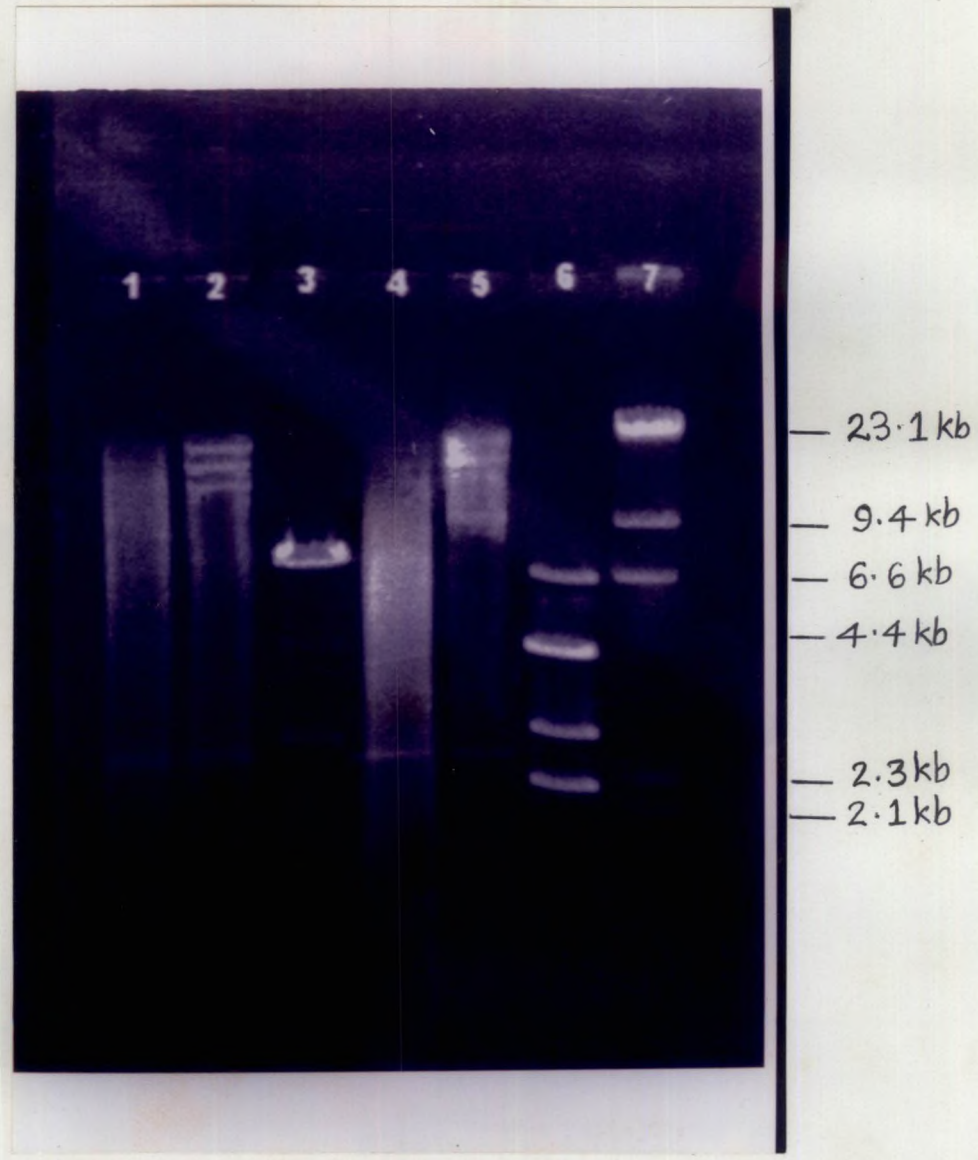
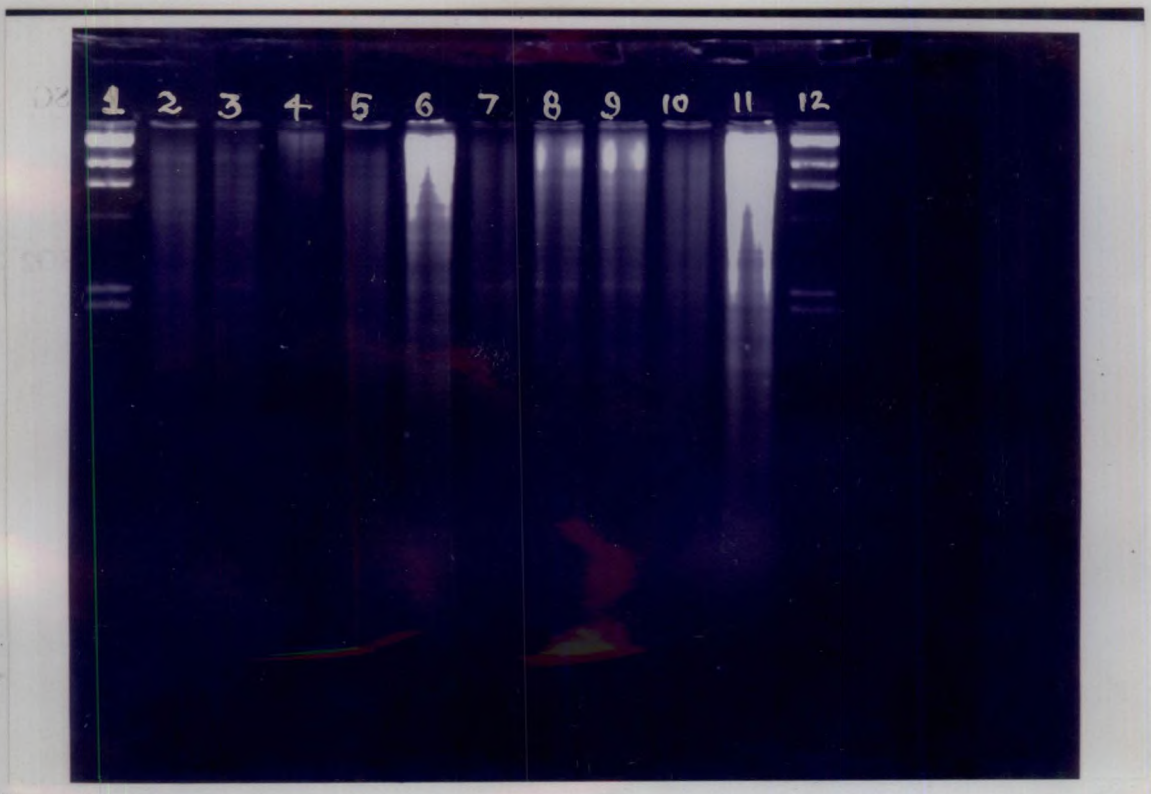

1
2
3
4
5
6
7
23·1 kb
9·4 kb
6·6 kb
4·4 kb
2·3 kb
2·1 kb

Fig.3 Restriction enzyme analysis of chromosomal DNA

Lane 1,12 : λDNA HindIII digest with standard size

Lane 2,3,4,5,6 : EcoRI digested genomic DNA of SE, SG1, SG2, ST and SB isolates

Lane 7, 8,9,10,11 : Hind III digested genomic DNA of SE, SG1, SG2, ST and SB isolates


1 2 3 4 5 6 7 8 9 10 11 12

Discussion

5. DISCUSSION

5.1 Identification

Cowan (1974) described certain primary tests for the identification of salmonella viz., catalase, oxidase, production of acid from glucose and oxidative/fermentative utilisation of glucose. The salmonellae serotypes obtained were Gram negative bacilli, catalase positive and oxidase negative. They grew aerobically and anaerobically and fermented glucose. They were also found to be non hemolytic on blood agar.

In the second stage biochemical tests, the two non motile salmonellae gave positive reactions in maltose and dulcitol and negative for arginine dihydrolase, ornithine decorboxylase activity and gas production. The results obtained in the present study clearly indicated that the non motile salmonellae were none other than *S. gallinarum* and concur fully with the findings of Cowan (1974); Pomeroy (1984), Snoeyenbos (1984) and Ashton (1990).

The results of the second stage biochemical and sugar fermentation tests of paratyphoid organisms viz., SE, ST and SB were identical and are in confirmity with the characters described for the particular serotypes by Holt *et al.* (1994).

5.2 Serotyping

S. enteritidis, *S. typhimurium* and *S. branderup* were serotyped at the National Escherichia and Salmonella Centre, Kasauli H.P. Both non motile

isolates were serotyped as *S. gallinarum* as they gave positive agglutination reactions with the polyvalent O antisera and factor sera 9 and 12 in plate agglutination test and negative reations with factor sera 2; 4; 7; 8; 3,10; 1,3,19; 11; 13; and 6. This finding concurs with the observations of Ewing, (1986).

5.3 Antibiogram

Antibiogram studies revealed that all the isolates were sensitive to ampicillin, ciprofloxacin, cotrimazine, enrofloxacin, streptomycin, kanamicin, nalidixic acid, neomycin and nitrofurantoin.

All serotypes were multiple drug resistant. SB was quadruple resistant (CxDOR), SG1 and SG2 were quintuple resistant (ChCxRSzS3), SE was sextuple resistant (ChCxGRSzS3) and ST was septuple resistant (ChCCxFRSzS3).

In the present study resistance to sulpha drug was common except in *S. branderup*. A similar finding had been reported in *S. typhimurium*, *S. enteritidis* and *S. gallinarum* by Threlfall *et al.* (1989); Poppe *et al.* (1993) and Anjanappa *et al.* (1993) respectively.

High percentage of multiple drug resistance in isolates from avian sources had been reported by Poppe and Gyles (1987) and Oh and Choi (1994). The present findings fully agree with the observations of Poppe and Gyles (1987) and Oh and Choi (1994). In the present study four different resistance patterns could be observed using 20 antibiotics/ chemotherapeutics. Resistance of salmonellae to

antimicrobial substances had commonly been used to subdivide salmonella serovars and for epidemiological purposes (Grand *et al.*, 1977).

5.4 Plasmid profile

The plasmid content of each of the five isolates of avian salmonellae was analysed on agarose gel electrophoresis. The number of plasmids in three serotypes varied from two to three and two serotypes did not show the presence of any plasmids. The size of the plasmids ranged from 1.75 kb to 48.32 kb.

SE and SB did not exhibit any plasmid carriage though they were multiple drug resistant like any other strain. Nair *et al.* (1995) reported that 84 per cent of *S. enteritidis* isolates from chicken, eggs and environment though plasmid-less expressed resistance to one or more of the antimocribial agents. The present findings are in agreement with the observations of Nair *et al.* (1995).

Both SG1 and SG2 had two low molecular weight plasmids of equal size, but the presence of a high molecular weight plasmid on SG1 did not confer any additional detectable resistance character to the bacteria. Avila and Dela Cruz (1988) opined that the plasmids of 30 kb and above are large plasmids but Lax *et al.* (1994) suggested that 54 to 98 kb size plasmids can only be considered as virulence plasmids as they represent upto two per cent of the genetic information of the salmonella genome. The largest plasmid detected in the present study was 48.32 kb in SG1 which is also far below the minimum size of virulence plasmid as observed by Lax *et al.* (1994).

ST has two low molecular weight plasmids of 13.62 kb and 4.2 kb. Poppe and Gyles (1987) reported the presence of low molecular weight plasmids in high per cent of *S. typhimurium* isolated from avian sources. Plasmid mediated sulfonamide and Trimethopim resistance in *S. typhimurium* had been reported by Threlfall *et al.* (1996). The results of present study on plasmid profile are consistent with those of Poppe and Gyles (1987) and Threlfall *et al.* (1996).

In the present study, plasmid profile analysis could differentiate the two strains of *S. gallinanum* where antibiogram could not. Hence identification of salmonella strains by their plasmid profile was found to be more accurate than antimicrobial susceptibility testing. This finding concurs with those of Holmberg *et al.* (1984).

5.5 Restriction enzyme analysis of plasmid DNA

As SG1, SG2 and ST had different plasmid profile pattern, the fragments generated after restriction enzyme digestion could not be compared as it is being done for identical plasmid profile pattern. Both SG1 and SG2 had two low molecular weight plasmids of equal size. Price *et al.* (1993) suggested that the presence of a similarly sized plasmid in the strains might belong to the same family and the hypothesis could be confirmed by restriction mapping. The comparison could not be made as one plasmid of size 2.6 kb was uncleaved and the fragments of the other plasmid (1.8/1.75 kb) could not be detected on the gel as it was cleaved into smaller size fragments that had migrated out of the gel.

In ST also, 1.5 kb and 1.24 kb of plasmid DNA after EcoRI and HindIII digestions respectively could not be detected on the gel as it might have been cleaved into low molecular size fragments that had migrated out of the gel.

A search of literature did not reveal the restriction enzyme digestion pattern for low molecular weight plasmids found in salmonellae.

5.6 Curing of plasmids

5.6.1 Curing with EB

In the present work, EB was found to eliminate small as well as large plasmids from salmonellae, Jones *et al.* (1982) obtained curing in *S. typhimurium* by treating it with EB, SDS or novobiocin. Nakamura *et al.* (1985) eliminated 36 Mdal plasmid present in *S. enteritidis* by culturing at 42°C overnight with ethidium bromide. Poppe and Gyles (1988) and Verma (1988) also obtained substantial degree of elimination of plasmids with EB. So the results of present study agree perfectly with the observations of Jones *et al.* (1982); Nakamura *et al.* (1985); Poppe and Gyles (1988) and Verma (1988).

5.6.2 Curing with acridine orange

Curing of plasmids did not occur with acridine orange in all the three isolates. Similar findings were reported by Hahn and Ciak (1976) and Poppe and Gyles (1988).

5.6.3 Curing with SDS

SDS has not eliminated the plasmids present in all the three salmonellae. The present work concurs with the findings of Poppe and Gyles (1988).

5.6.4 Curing at elevated temperature

Incubation of salmonellae at 45°C resulted in the elimination of plasmids present in the three isolates. The present findings are in accordance with those of Barrow *et al.* (1987), Poppe and Gyles (1988), Anjanappa *et al.* (1993).

5.7 Pathogenicity studies

Both SE and SB were devoid of any plasmids, hence only the wild isolates were given to assess the pathogenicity of the organism in day old chicks. Serotype, SE killed all the birds within 72 hours when inoculated by oral and intraperitoneal routes which indicated that the plasmidless serotypes were virulent. But Helmuth *et al.* (1985) had observed that plasmid-less *S. enteritidis* strains were less virulent. The results of present study could not agree with the findings of Helmuth *et al.* (1985).

ST cured of 13.62 kb and 4.2 kb plasmids did not produce any death in chicks challenged intraperitoneally. It clearly indicated that these plasmids were responsible for virulence. The results of this study concur with the findings of Poppe and Gyles (1987) and Barrow and Lovell (1988). But cured and wild isolates of ST did not make much difference in causing death of chicks challenged

orally. The reason may be that the absence of an established gut flora in day old chicks which would allow massive microbial multiplication from small inocula and that makes the oral LD50 of newly hatched chicks unreliable. The results of the present study agree with the findings of Barrow *et al.* (1989).

Both wild and cured strains of SG1 and SG2 did not produce any clinical signs/death in the day old chicks challenged orally or intraperitoneally and it indicated that day old chicks are refractory to infection by both routes. This study necessitates the use of other experimental animals/routes.

5.8 Restriction enzyme analysis of chromosomal DNA (REAC)

Indistinct separation of bands were observed with each isolate digested with EcoR1 and HindIII enzymes. Blackall *et al.* (1995) reported the similar difficulties with EcoRI enzyme. It was difficult to distinguish the fragments obtained by endonuclease digestion of chromosomal DNA from each other and this made the technique unworthy. Similar finding was made by Wasteson *et al.* (1992).

Summary

5. SUMMARY

Two *S. gallinarum* and three paratyphoid organisms viz., *S. enteritidis*, *S. typhimurium* and *S. branderup* of avian origin were initially identified by morphological, cultural and biochemical tests and then by serotyping.

All the isolates showed multiple drug resistance ranging from four to seven drugs by agar diffusion test. There was cent per cent sensitivity to ampicillin, ciprofloxacin, cotrimazine, enrofloxacin, kanamicin, nalidixic acid, neomycin, nitrofurantoin and streptomycin. Resistance to cephalothin, cloxacillin, rifampicin, sulfadiazine and triple sulphas was common among four of the isolates. Four different resistance pattern was observed among five avian salmonellae. Though antibiogram was used as an epidemiological marker in the past, it was found to vary depending upon the number of antibiotics used. So antibiogram could only be used as an adjunct to plasmid profiling in epidemiological studies.

Analysis of the 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 kb to 48.32 kb. *S. enteritidis* and *S. branderup* were devoid of any plasmids. Among the three plasmid positive strains, three different plasmid profile pattern were obtained. Two identical low molecular weight plasmids were present in both *S. gallinarum* isolates. One had an additional high molecular weight plasmid of 48.32 kb. These

two isolates had identical antibiogram pattern, but plasmid profile could differentiate these two efficiently. The plasmid profiling could be a valuable epidemiological tool to differentiate and identify the various isolates of salmonellae.

Three different restriction pattern could be observed after digestion of plasmids with EcoRI and HindIII separately. The large plasmid was cleaved by EcoRI and HindIII yielding fragments of total size 48.32 kb for each of the enzymes. One plasmid of 2.6 kb was uncleaved and the cleaved fragments of other plasmid of 1.75 kb could not be detected on the gel indicating that it may be cleaved into extremely low molecular size DNA fragments. Restriction enzyme analysis provides a measure of plasmid relatedness between the various salmonellae.

Ethidium bromide at 100 µg/ml was found to be the best curing agent among the three chemicals used. Acridine orange and sodium dodecyl sulphate had no effect in curing the plasmid DNA from the salmonellae after one and three days of incubation respectively. Exposure to elevated temperature at 45°C was a better physical curing method and the curing was obtained only after two weeks of incubation. Loss of the plasmids resulted in loss of resistance character only in *S. typhimurium* indicating the plasmid borne nature of resistance.

In the pathogenicity studies, all day old chicks were killed within 72 hours of oral administration and intraperitoneal inoculation of plasmid-less *S. enteritidis* and *S. branderup*. *S. typhimurium* cured of two plasmids was less pathogenic to

the chicks when compared to wild strains. Intraperitoneal inoculation was found to be the better route than the oral administration. Day old chicks were found to be refractory to infection to *S. gallinar um* by both the routes.

Restriction enzyme analysis of chromosomal DNA with EcoRI and HindIII enzymes yielded indistinguishable band pattern which could not be compared.

Plasmid profiling can be used as a rapid, sensitive, economical, reproducible tool in differentiating avian salmonellae than restriction enzyme analysis of chromosomal DNA.

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MOLECULAR CHARACTERISATION OF SALMONELLAE ISOLATED FROM POULTRY

By

G. TRESSA MARY

ABSTRACT OF A THESIS

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Master of Veterinary Science

Faculty of Veterinary and Animal Sciences

Kerala Agricultural University

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR - 680651

KERALA

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ABSTRACT

Five serotypes of salmonellae from avian sources were examined for biochemical properties, serology, drug resistance, plasmids, restriction enzyme pattern of plasmid as well as genomic DNA and pathogenicity.

The biochemical characters are in confirmity with the characters described for the serotypes by the earlier workers.

The study of antibiogram with 20 antibiotics/chemotherapeutic agents revealed the presence of multiple drug resistance in all the five serotypes.

In the plasmid analysis, *S. enteritidis* and *S. branderup* were found to be plasmid free. The number of plasmids in other serotypes ranged from two to three and the size ranged from 1.75 kb to 48.32 kb. Identical low molecular weight plasmids were present in both *S. gallinarum*. The presence of large plasmid in one of the *S. gallinarum* did not confer any additional detectable resistance character. *S. typhimurium* contained two plasmids of sizes 13.62 kb and 4.2 kb.

Restriction enzyme analysis of plasmid DNA from three salmonellae with EcoRI and HindIII yielded three different restriction pattern with each enzyme.

Ethidium bromide and exposure to elevated temperature cured the plasmids present in the salmonellae within two days and 14 days respectively. Acridine orange and sodium dodecyl sulphate were found to be ineffective in curing the

plasmid DNA. The elimination of plasmids resulted in the loss of resistance to antibiotics was demonstrated in *S. typhimurium*.

In tests to assess the differences in pathogenicity between wild and cured isolates of *S. typhimurium* in day old chicks, only intraperitoneal route was found to be effective when compared to oral route. A relation of plasmids to virulence was noted only in *S. typhimurium*. Day old chicks were refractory to infection to *S. gallinarum* by both the routes.

Plasmids encoding both resistance and virulence were observed in *S typhimurium*. Plasmid negative serotypes of *S. enteritidis* and *S. branderup* were found to be equally virulent as wild strains of *S. typhimurium*. So a definite correlation between virulence and plasmids could not be made.

Restriction enzyme analysis of chromosomal DNA yielded bands which were indistinct and so uncomparable. Hence of the tests based on the analysis of genetic content plasmid profile was found to be efficient in typing the isolate rather than restriction enzyme analysis of genomic DNA.