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Epidemiological Characterization of Avian *Salmonella enterica* Serovar Infections in India

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Abstract: The study was undertaken to elucidate the recent epidemiological status of salmonellosis in India. A total of 23 *Salmonella* isolates were recovered from different disease outbreaks in different geographical locations. The phenotypic analysis by serotyping, transmission electron microscopy and antibiogram profiles were able to classify the *Salmonella* isolates based on different serovars. The *S.* Gallinarum isolates were also classified into different strains based on the resistance pattern to different antimicrobials used. The plasmid profiles of the *Salmonella* isolates were also found to be serovar specific and the *S.* Gallinarum isolates were subdivided into different strains based on the geographical origin. There was a positive correlation between the antibiotic resistance pattern and the presence of plasmid within different serovars.

Key words: *Salmonella*, transmission electron microscopy, antibiogram profiles, plasmid profiles

Introduction

Salmonellosis is a hyperendemic disease in India affecting both man and animals alike. There are over 2464 serovars of salmonella (Popoff and Minor, 1997), distributed widely in nature, and in India, more than 235 serovars have been recorded, and this number is increasing constantly (Reeves *et al.*, 1989). Salmonellosis in poultry caused by *Salmonella enterica subsp. enterica* serovars Gallinarum (biovars Gallinarum and Pullorum), and Enteritidis is the leading cause of morbidity and mortality in poultry and responsible for significant economic loss in all the phases of poultry industry from production to marketing. Salmonellosis treated with antibiotics or other chemotherapeutic agents often resulted in the development of resistant strains, due to transfer of drug resistance through episomal transfer of resistance factors posing a great threat to public health.

There is a paucity of information about the reason for the increased prevalence of salmonellosis in poultry, the possibility that the increased occurrence of *Salmonella* infection may be due to change in the characteristics of the pathogen. The study of pathogen transmission requires precise determination of the degree of genetic relatedness among isolates, so that identification of strains causing the disease is essential for epidemiological investigation of *Salmonella* outbreaks. Biotyping, phage typing and antimicrobial resistance pattern have been used as phenotypic epidemiological markers for *Salmonellae* since long, but they have their own limitations (Edelman and Levine, 1986). Of late, plasmid profiles have also become valuable markers in the epidemiological investigation (Ferris *et al.*, 1992;

Joseph *et al.*, 1997). Since the prevalence of plasmids in *Salmonella* species (Taylor *et al.,* 1982), there have been many reports that plasmid profiles was useful as an epidemiological tool in outbreaks of salmonellosis (Riley and Cohen, 1982; Taylor *et al.*, 1982; Riley *et al.*, 1983).

Thus, the aim of the present investigation was to study the prevalence of salmonellosis and characterize the isolates based on electron microscopy, antibiogram and plasmid profiles variability in the serovars and strains obtained from different geographical locations to check the epidemiological relationship.

Materials and Methods

Reference *Salmonella* strains: Standard *Salmonella* Gallinarum 9R (SG-9R) and *Salmonella* Pullorum strains were procured from Indian Veterinary Research Institute, Izatnagar, U.P, India and Institute of Animal Health and Veterinary Biologicals, Bangalore, Karnataka, India respectively and were maintained as pure cultures in the laboratory.

Salmonella strains recovered from outbreaks: *Salmonella* strains were isolated from diseased birds in different poultry breeding farms. A total of 108 samples were collected from 40 ailing and dead birds in the range of day one to 60 weeks of age and ten dead in shells. The isolation and identification were carried out as per Mallinson and Snoeyenbos. (1989), and after confirmation as *Salmonella* they were sent to National *Salmonella* and *Escherichia* center, Central Research Institute, Himachal Pradesh, India, for serotyping. Prefectures (regions) in which the *Salmonella* were

isolated in India were from the states of Karnataka, Maharastra and Tamilnadu.

Transmission electron microscopy: The standard two step drop method was followed as described by Biel and Gelderblom (1999). A drop of bacterial suspension was placed on parafilm and a 200 mesh copper grid (M/s Sigma, USA) with a film reinforced with carbon was placed on bacterial suspension for two minutes and excess material was wicked away with an edge of filter paper. The grid was then washed with sterile distilled water to reduce the number of adsorbed cells. The coated grid with sample adsorbed to the surface was stained with 2% uranyl acetate for two minutes. The grids were then air dried and loaded on to cartridge and screened under transmission electron microscope (Joel 100S, M/s Joel Ltd., Japan) under different magnifications.

Antibiogram assay: All the isolates including the standard reference *Salmonella* strains were subjected to drug sensitivity test using 16 different antimicrobial agents by disc diffusion method as described by Miles and Amyes (1996) and the interpretation was made as per the zone size interpretation chart provided by the manufacturer of antibiotic discs (Himedia, India). The antibiogram profiles were categorized into four groups based on sensitivity pattern and was scored as highly resistant (0), less sensitive (1), moderately sensitive (2) and highly sensitive (3) and the scored data were used for statistical analysis.

Plasmid profile analysis: Plasmids were detected according to the method described by Kado and Liu (1981). Bacterial cells were grown overnight in 10 ml of Luria Bertani broth at 37°C, harvested by centrifugation, and suspended in 1 ml of E buffer (40mM Tris-acetate, 2mM EDTA[pH 7.9]). The cells were then lysed by the addition of 2ml of freshly prepared lysing solution (3g of sodium dodecyl sulfate, 0.6g of Tris, and 6.4 ml of 2N NaOH in 100 ml of distilled water), incubated for 1 hr at 55°C, and extracted with 6 ml of phenol-chloroform (1:1 v/v). After centrifugation, the supernatant was subjected to agarose gel electrophoresis in 0.7% agarose with Tris-borate-EDTA buffer and stained with ethidium bromide (1μg/ml) for detection and sizing of plasmid DNA. Plasmid bands were viewed by UV transilluminator and recorded in gel documentation system (AlphaImager 2200, USA). The molecular weight of plasmids was determined using gel documentation software by comparing the known molecular weight DNA marker (Lambda/*Hind* III fx174/*Ha*e III marker) (M/s Biogene, USA). Based on the molecular size, the plasmids were scored for the presence (1) or absence (0) in all the isolates studied and the binary data were used for the statistical analysis.

Statistical analysis: The antibiogram profile scored data and the binary data of plasmid profiles were analyzed with the computer package STATISTICA. The dissimilarity matrix was developed using Squared Euclidean Distance (SED) that estimated all pair wise differences (Sokal and Sneath, 1973) and dendrogram was computed based on Ward's method of clustering. The dendrogram was also computed by combining the plasmid profiles and the antibiogram profiles to check the correlation between antibiotic resistance and the presence of plasmids.

Results

Prevalence of salmonellosis: 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 Tamilnadu respectively.

All the isolates were confirmed to their O: H: K serotypes, fell under five categories viz., *Salmonella* Gallinarum (16), *Salmonella* Enteritidis (5), *Salmonella* Typhimurium (1), and *Salmonella* Worthington (1). The most predominant serotype was *S.* Gallinarum accounting for 69.6% followed by *S.* Enteritidis for 21.7% (Table 1).

Transmission electron microscopy: The electron microscopy of the *Salmonella* serovars had rod shaped morphology with presence of microstructures such as peritrichous flagella and pili external to the cell wall (Fig. 1). The standard strain SG-9R (Fig. 1a) appeared as long slender rods, much bigger than *S.* Pullorum and *S.* Gallinarum isolates (Fig 1b, 1c), and the flagella and pili of all these isolates were not much prominent.

The *S.* Enteritidis, *S.* Typhimurium and *S.* Worthington isolates (Fig. 1d, 1e, 1f) were also having rod shaped morphology but much bigger than that of the *S.* Gallinarum serovars with presence of very prominent flagella and pili in larger numbers. The flagella and pili of the *S.* Enteritidis isolates were much larger and prominent among all the serovars screened.

Antibiogram assay: The dendrogram computed on 25 *Salmonella* isolates based on the antibiogram sensitivity pattern (Fig. 2), clearly divided the accessions into two major clusters. The maximum dissimilarity distance between the two major clusters was 240 units. The cluster A consisted of all the strains of *S.* Gallinarum and *S.* Pullorum, which were resistant to sulphas (i.e., sulpha diazine and sulphamethoxy pyridazine), neomycin and furazolidone. This cluster can be grouped into two subclusters A1 and A2 and the dissimilarity between the two subclusters was 45 units. The subcluster A1 consisted of 13 individuals, which again can be grouped into two subgroups A1.1 and A1.2 with a linkage distance of 25 units. In the subgroup A1.1, all
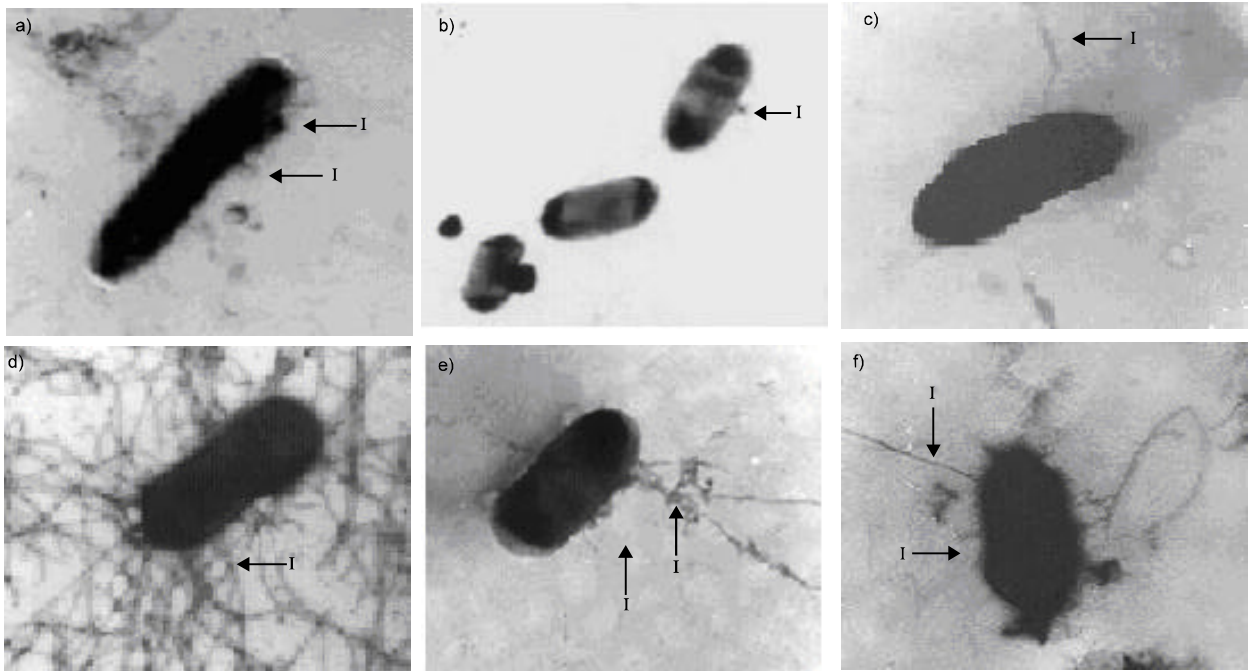
Fig. 1: Transmission electron microscopy of *Salmonella* serovars, having rod shaped morphology with the presence of peritrichous flagella (F) and pili (P).
a) *S*. Gallinarum-9R (21,000X), b) *S*. Pullorum (21,000X), c) *S*. Gallinarum field isolate (21,000X), d) *S*. Enteritidis (10,000X), e) *S*. Typhimurium (10,000X), f) *S*. Worthington (10,000X).
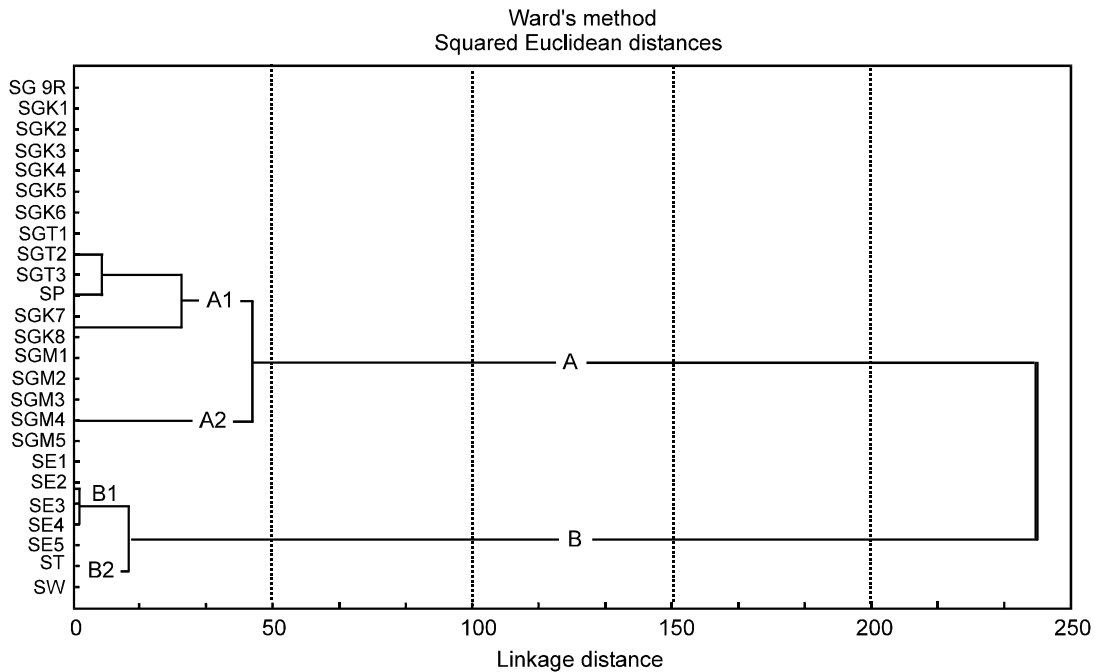

Fig. 2: Dendrogram computed based on the antibiogram profiles of *Salmonella* isolates

the *S*. Gallinarum isolates from Karnataka, Tamilnadu and *S*. Pullorum were clustered except SGK7 and SGK8 belong to this subgroup A1.2 that were less sensitive to ciprofloxacin, when compared to subgroup A1.1. The subcluster A2 consisted of the *S*. Gallinarum isolates from Maharastra, which were resistant to enrofloxacin
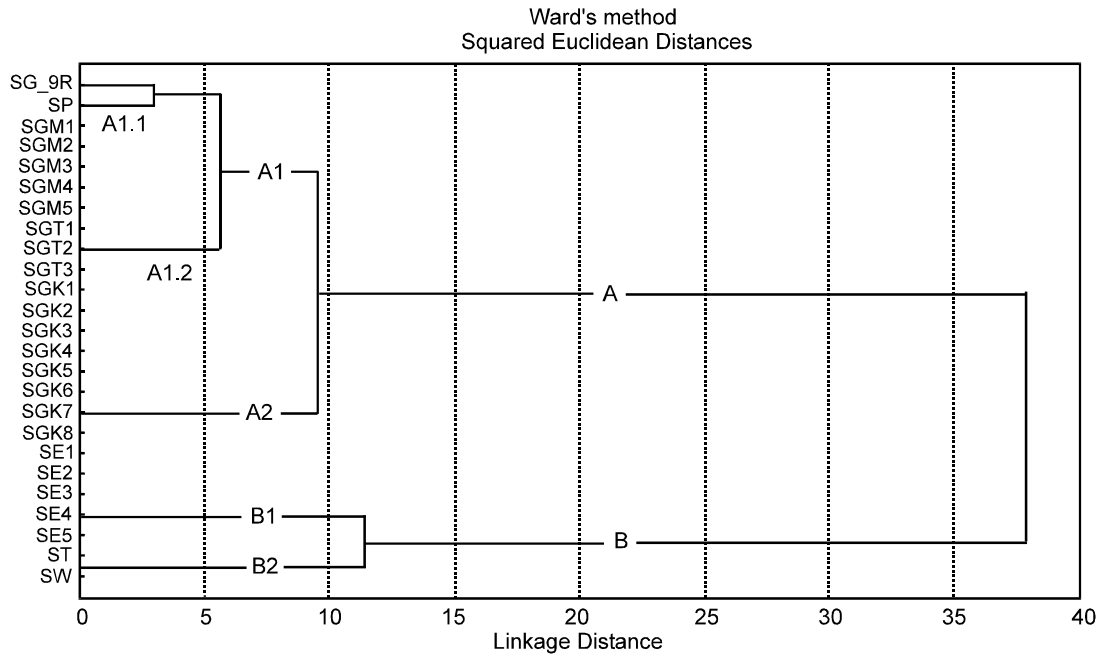
Ward's method
Squared Euclidean Distances

Fig. 3: Dendrogram computed based on the Plasmid profile of *Salmonella* isolates

and sensitive to ciprofloxacin, when compared to the above subcluster A1.

The cluster B was resistant to furazolidone, nitrofurantoin, colistin, ciprofloxacin and neomycin, when compared to cluster A. The cluster B was again divided into two subcluster B1 and B2 at a distance of 20 units. The subcluster B2 consisted of *S.* Typhimurium and *S.* Worthington isolates, which were less sensitive to ampicillin and cloxacillin, when compared to subcluster B1, which consisted of *S.* Enteritidis isolates.

Plasmid profiles analysis: The dendrogram computed based on plasmid profiles of 25 *Salmonella* isolates (Fig. 3) clearly divided the accessions into two major clusters and the maximum dissimilarity distance between the clusters was 37 units. The cluster A consisted of all the isolates of *S.* Gallinarum and *S.* Pullorum which had common plasmid of 26.5 kb, 11.8 kb and 4.5 kb. This cluster can be further grouped into two subclusters A1 and A2 and the dissimilarity between the two subclusters was 10 units. The subcluster A1 consisted of 10 individuals, which again can be grouped into two subgroups A1.1 and A1.2 with a linkage distance of 5 units. In the subgroup A1.1 consisted of SG-9R and *S.* Pullorum, which had 5.5 kb in common, which is absent in the other *S.* Gallinarum strains. However SG-9R had 1.5kb plasmid, which was lacking in *S.* Pullorum. The isolates of subcluster A1 consisting of all the *S.* Gallinarum isolates from Karnataka lacked 17.4 kb plasmid when compared to other subcluster A2. The cluster B was again divided into two subclusters B1 and B2 at a distance of 12 units. The subcluster B2

consisted of *S.* Typhimurium and *S.* Worthington isolates, which had 6.6 kb and 28.2 kb plasmids, which were lacking in all other isolates and subcluster B1, consisted of *S.* Enteritidis isolates with a 24.2 kb plasmid, which was absent in other isolates.

The principal component analysis based on two factors (Fig. 4) grouped the isolates into two main clusters, wherein the first cluster consisted of the *S.* Enteritidis, and the second cluster consisted of *S.* Gallinarum. The *S.* Pullorum was placed near the second cluster. The *S.* Typhimurium and *S.* Worthington isolates were placed away from these two main clusters. The cluster of *S.* Gallinarum isolates analyzed separately were further grouped into three clusters (Fig. 5), wherein the first cluster consisted of Karnataka isolates, and the second consisted of Maharastra and Tamilnadu isolates and the SG-9R strain was placed away from these two clusters. Correlation between plasmid and antibiogram profiles

The dendrogram computed by combining the data to correlate with the presence of plasmid and antiboigram pattern (Fig. 6), divided the accessions into two major clusters. The whole dendrogram was distributed between SG-9R to *S.* Worthington and the maximum dissimilarity distance between the two major clusters was 275 units. The maximum dissimilarity of 33% was found between *S.* Worthington and *S.* Gallinarum (SG-9R, SGK7 and SGK8). The maximum dissimilarity of 10% within the *S.* Gallinarum isolates was among SGK7, SGK8 and SG-9R.

There was a positive correlation within *Salmonella* serovars, where the *S.* Enteritidis isolates in cluster B, had 24.2 kb plasmid and they were resistant to colistin,

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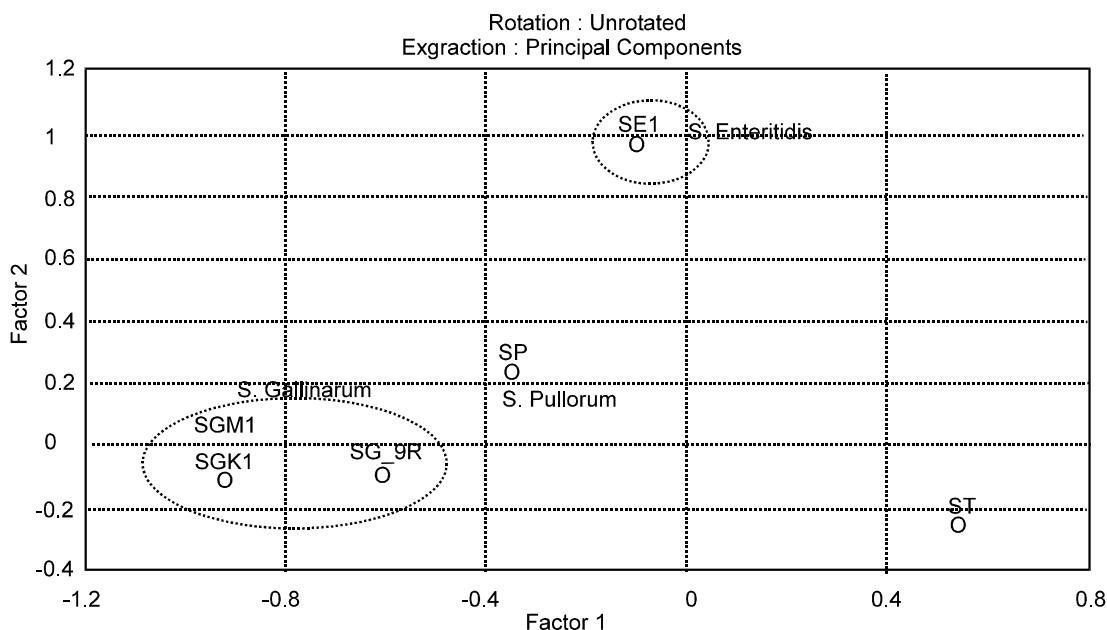Exgraction : Principal Components

Fig. 4: Principal component analysis based on the plasmid profiles of *Salmonella* isolates.

ciprofloxacin, enrofloxacin, neomycin and nitrofurantoin antibacterials, when compared to the isolates of cluster A. The *S*. Typhimurium and *S*. Worthington isolates had 21.1 kb and 6.6 kb plasmids that were resistant to ampicillin and cloxacilin antibacterials, when compared to the *S*. Enteritidis isolates. But, there was no positive correlation within different *S*. Gallinarum strains isolated, but on the contrary the *S*. Gallinarum isolates from Maharastra lacking 17.4 kb plasmid were resistance to enrofloxacin.

Discussion

The word clone is used to denote bacterial cultures isolated independently from different sources, in different locations, and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin (Orskov and Orskov, 1983). It is necessary in each case to define the level of identity which will depend on the method used, their discriminatory power, reproducibility and typability and on the diversity of strains found in the area of interest.

The present study on seroprevalence of avian *Salmonella* has led to the conclusion that certain serovars of *S*. Gallinarum and *S*. Enteritidis are more predominant, when compared to the other serovars of *Salmonella,* accounting to 69.6% and 21.7% respectively, confirming the earlier findings (Legutko. 1995; Woo *et al.*, 2000) of fowl typhoid distributed throughout the world (Shivaprasad, 2000).

Salmonella Gallinarum and Pullorum are highly adapted to avian species and is host specific and of little public

health significance. Serotypes such as *S*. Typhimurium and *S*. Enteritidis recovered, have been predominated in the poultry industry and human illness for many years (Guinee and van Leeuwen, 1978). The *S*. Worthington isolated is an unusual isolate having antigenic structure of 1, 13, 23: z: lw, belonging to serogroup G. It was isolated for the first time in 1937 from animals. Since then, it has been isolated from other animals and human sources (Mahajan *et al.*, 1997). Newborn babies, especially premature appear to be more susceptible than the general population and the close proximity with each other facilitates the spread of infection (Ayyagari *et al.*, 1990). Isolation of the *S*. Enteritidis, *S*. Typhimurium and *S*. Worthington serovars in the present study is an indication of a close association of man and poultry during any of the farm operations or consumption. Moreover, these birds may serve as potential source of infection to man causing a public health problem.

The cell wall is responsible for many of the taxonomically significant features of bacterium, their shapes, and their major divisions into gram-positive and gram-negative organisms, and antigenic specificities that are important in classification and in the interaction of pathogens with the host (Gyles and Thoen, 1993). The *Salmonella* serovars have a sheath surrounding the flagellum; this sheath is continuous with the outer membrane of the gram-negative cell wall. The transmission electron microscopy has increased the phenotypic classification of the *Salmonella* serovars by the size, shapes and the presence of the peritrichous flagella (Fig. 1). Jones *et al.* (1992) have recently shown increased invasiveness of chemotaxis mutants of *S*. Typhimurium that were smooth swimmers and
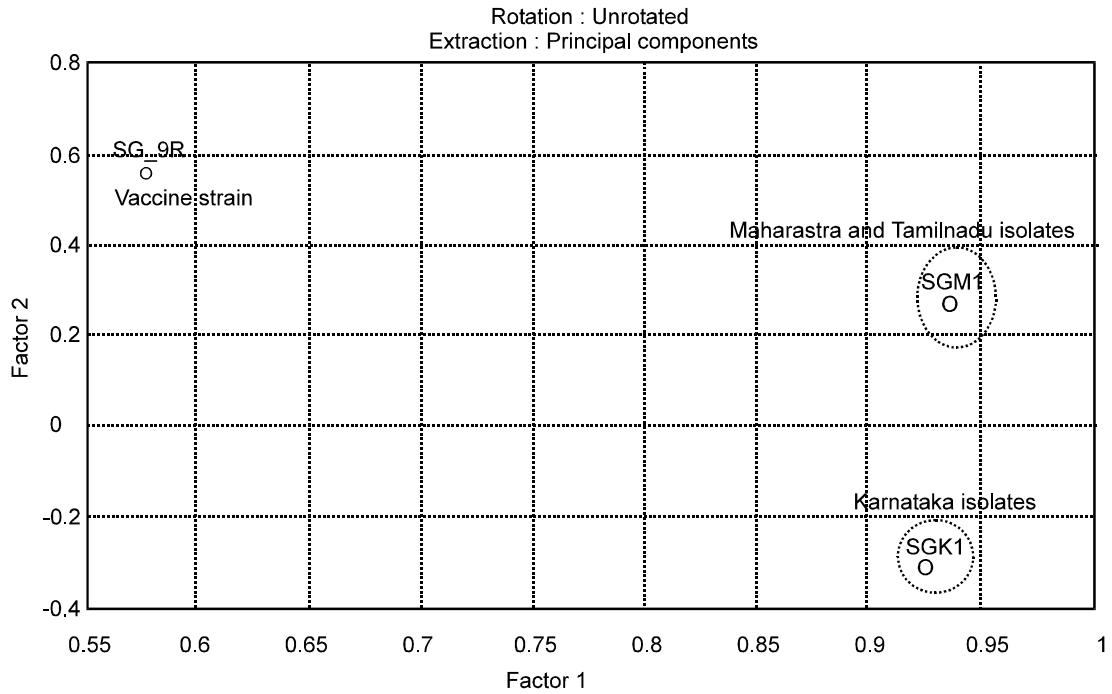
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Extraction : Principal components

Fig. 5: Principal component analysis based on the plasmid profiles of *Salmonella* Gallinarum isolates
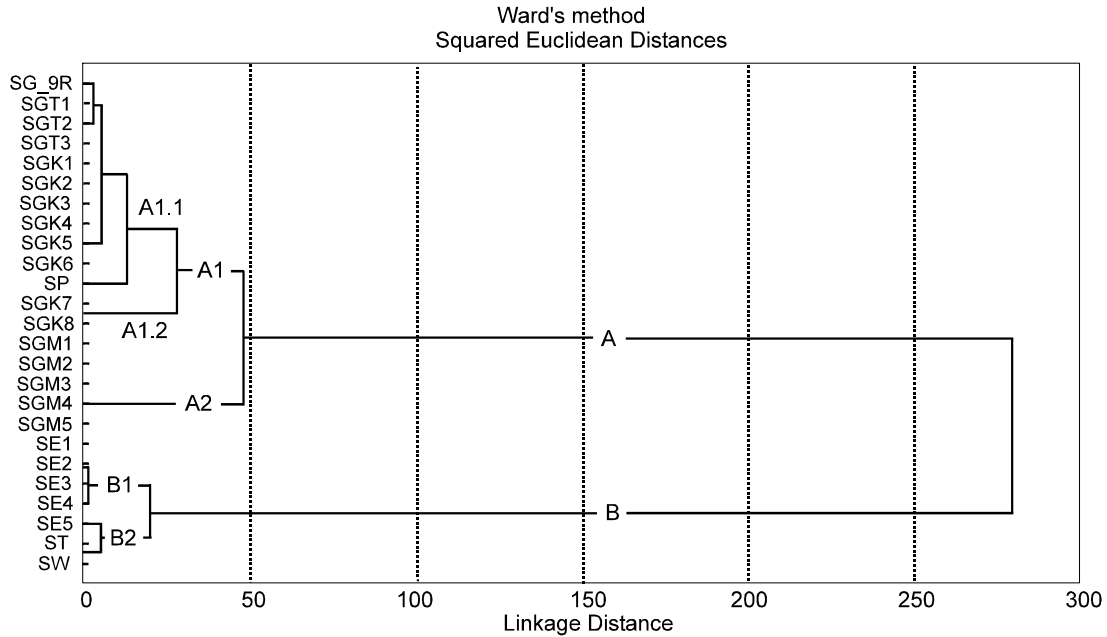
Ward's method
Squared Euclidean Distances

Fig. 6: Dendrogram computed based on antibiogram and plasmid profiles of Salmonella isolates

decreased invasiveness of tumbly mutants. Smooth swimmers have their flagella organized into a bundle at one pole, so that most of the bacterial surface is free of projecting flagella; tumbly swimmers have their flagella projecting from most of the bacterial surface, which was noticed in our study.

The antibacterials used were selected based on their extensive use for either chemoprophylaxis or therapy for the control of bacterial diseases in poultry. The antibiogram profiles based on the sensitivity were able to classify the *Salmonella* isolates based on serovars viz., *S.* Gallinarum and *S.* Enteritidis (Fig. 2). There were minor variations in resistance to some antibacterials such as the Maharastra isolates were resistant to

Table 1: *Salmonella* serovars isolated from different disease outbreaks

I.D. No.	Serovar and antigenic structure	Source	No. of isolates	Abbreviation
1	*S.* Gallinarum rough strain (9,12,:-:-)	IVRI, Izatnagar, India	1	SG-9R
2	*S.* Pullorum	IAH & VB, Bangalore, India	1	SP
3	*S.* Gallinarum (9,12,:-:-)	Karnataka	8	SGK1, SGK2, SGK3, SGK4, SGK5, SGK6, SGK7, SGK8
		Maharastra	5	SGM1, SGM2, SGM3, SGM4, SGM5
		Tamilnadu	3	SGT1, SGT2, SGT3
4	*S.* Enteritidis (9,12:gm:-)	Karnataka	5	SE1, SE2, SE3, SE4, SE5
5	*S.* Typhimurium (4,5:I: 1,2)	Karnataka	1	ST
6	*S.* Worthington (1,13,23:z: lw)	Karnataka	1	SW

Enrofloxacin. The results of the antibiogram profiles, did not correlate exactly with those of earlier workers especially in case of resistance to tetracycline (Swaminathan, 2001; Batabyal *et al.*, 2003; Lee *et al.*, 2003), which may be due to the fact that, a particular antibiotic is effective at the time of introduction to the market later becomes ineffective once it is used inadvertently for a long duration in sub optimal doses. Hence, it is appropriate to select an antibiotic based on antibiogram pattern rather than earlier reports, because no single antibiotic would be effective at all times.

Bacterial plasmids contribute a wide variety of phenotypes to their bacterial host, including antibiotic resistance and virulence properties. Many analyses of *Salmonella* plasmids have concentrated on the epidemiology and molecular properties of plasmids originating from antibiotic resistant strains. These analyses have revealed that in a given geographic area an individual bacterial clone predominates (Van Embden *et al.*, 1976; Helmuth *et al.*, 1981). In the present study the plasmid profiles were able to classify the *Salmonella* isolates based on the taxonomy of the Genus, which is serovar specific (Fig. 3, 4) (Helmuth *et al.*, 1985; Brown *et al.*, 1986; Stefanova *et al.*, 2002). The *S.* Gallinarum isolates based on the plasmid profiles (Fig. 5) were subdivided into two different strains based on the geographical location excluding the SG-9R standard strain. The attempt made to correlate the plasmid profiles with antibiotic resistance, may be useful in epidemiological characterization (Radu *et al.*, 1997; Bhattacharya *et al.*, 2001) of *Salmonella* isolates. There was no correlation between plasmid profiles and antibiotic resistance pattern within the *S.* Gallinarum strains isolated from different geographic origin (Rahman, 1999), but there was a good correlation within different serovars. Thus, a correlation could be made with the resistance pattern to different antibacterials and the plasmid profiles. However, the presence of plasmids may also correlate with virulence factors (Singh *et al.*, 1996; Oh *et al.*, 2002).

The transmission electron microscopy study was able to differentiate between different serovars but not different strains, but the incidence of plasmids and antibiogram profiles reflect the strain variations occurring which can be a useful epidemiological tool in the investigation of *Salmonella* outbreaks. The molecular epidemiology should also be accompanied by restriction analysis and chromosomal DNA studies which may further detect strain variations.

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