CHARACTERIZATION OF LOCAL *SALMONELLA ENTERITIDIS* ISOLATES OF CHICKEN, EGG AND HUMAN ORIGIN

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Prof. Dr. Tayfur ÖZTÜRK
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Suzan Kıncal
Head of the Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in the scope and quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Gülay Özcengiz
Co-supervisor

Assoc. Prof. Candan Gürakan
Supervisor

Examining Committee Members

Prof. Dr. Ufuk Gündüz

Assoc. Prof. Dr. Candan Gürakan

Prof. Dr. Gülay Özcengiz

Prof. Dr. N. Gürdal Alaeddinoğlu

Prof. Dr. Cumhur Çökmüş

ABSTRACT

CHARACTERIZATION OF LOCAL *SALMONELLA ENTERITIDIS* ISOLATES OF CHICKEN, EGG AND HUMAN ORIGIN

İçgen, Bülent

Ph.D., Department of Biotechnology

Supervisor: Assoc. Prof. Dr. Candan Gürakan

Co-supervisor: Prof. Dr. Gülay Özcengiz

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The eighty-two local isolates of *Salmonella enteritidis* from human, chicken and egg were characterized for their antibiotic resistance patterns, plasmid profiles, phage types (PT), outer membrane proteins (OMP) and lipopolysaccharide (LPS) patterns. Antibiotic susceptibilities of the 82 local isolates were determined by the disc diffusion method. Ten egg isolates did have 3 different antibiotic resistance profiles. Twelve different antibiotic profiles were observed with the 21 chicken isolates and the 51 human isolates showed 14 different antibiotic profiles too. All the strains were sensitive to quinolones, aminoglycosides and carbapenems. On the other hand, tetracycline resistance was the most common in the isolates from three sources human, chicken and egg (58.8%, 71.4% and 70%), respectively. The plasmid profiles of the isolates were also determined and comparisons were made between the samples for the number of plasmids present and their molecular weights. There were 13 profiles seen among the 51 human isolates from Bursa and Ankara. Human isolates showed greatest relatedness with chicken isolates. The human, the chicken and the egg isolates were from the same regions of the country, that is Bursa and Ankara. There were 5 profiles seen among the 21 chicken isolates. The chicken isolates showed the greatest relatedness with egg isolates. On the other hand, the 10 egg isolates displayed 6 different plasmid profiles. A 54 kb plasmid was harboured by human, chicken and egg isolates of *S. enteritidis* with a frequency of 94.1%, 90.5% and 100%, respectively. The susceptibility of isolates to different bacteriophages revealed that PT4 predominated in *S. enteritidis* human and egg isolates. On the other hand, PT6 was common in chicken isolates. The isolates from three sources generally belonged to the phage types PT1, PT4, PT6 and PT7 and pointed to an epidemiological connection between the isolates from human, chicken and egg. Lipopolysaccharide (LPS) profiles of all the isolates were analyzed by Proteinase K digestion method and followed by SDS-PAGE. 24 % of the chicken, 40% of the egg and 80% of the human isolates were shown to have smooth-type LPS profile. OMP profiles of the 82 isolates were determined with the sarcosyl extraction method. Four different outer membrane protein profiles were identified with respect to number of proteins and their molecular weights, and defined as OMP-typeI, OMP-typeII, OMP-typeIII and OMP-typeIV, respectively. 69% of the human, 38% of the chicken and all the egg isolates displayed OMP-typeII profiles whereas 52% of the 21 chicken isolates gave OMP-type III profile. The antibiotic resistance patterns, plazmid, phage type, LPS and OMP profiles of *S. enteritidis* isolates were compared to the nonenteritidis enterics.

Keywords: *S. enteritidis*, Epidemiology, Drug resistance (antibiogram) typing, Plasmid profile typing, Phage typing (PT), Lipopolysaccharide (LPS) profile, Outer membrane protein (OMP) profile, Principle component analysis (PCA).

ÖZ

İNSAN, TAVUK VE YUMURTA KÖKENLİ LOKAL *SALMONELLA ENTERITIDIS* İZOLATLARININ KARAKTERİZASYONU

İçgen, Bülent

Doktora, Biyoteknoloji

Tez Yöneticisi: Doç. Dr. Candan Gürakan

Yardımcı Tez Yöneticisi: Prof. Dr. Gülay Özcengiz

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Bu çalışmada insan, tavuk ve yumurta kökenli 82 *Salmonella enteritidis* lokal izolatı antibiyotik dirençlilik, plazmit, faj tipleri, dış zar proteinleri ve lipopolisakkarit profilleri bakımından karakterize edilmiştir. İzolatların antibiyotik dirençlilik profilleri disk diffüzyon metodu ile test edilmiştir. Buna göre 10 yumurta izolatı 3, 21 tavuk izolatı 12 ve 51 insan izolatı 14 değişik antibiyotik dirençlilik profili göstermiştir. Bütün izolatların quinolone, aminoglycoside ve carbapenem türü antibiyotiklere karşı duyarlı oldukları tesbit edilmekle birlikte, tetrasikline karşı insan izolatlarında %58.8, tavuk izolatlarında %71.4 ve yumurta izolatlarında yüzde 70 oranında dirençlilik olduğu gözlenmiştir. 82 *S. enteritidis* lokal izolatlarındaki plazmid DNA profilleri analiz edilmiş, her bir izolat için plazmidlerin sayısı ve molekül ağırlıkları bakımından karşılaştırma yapılmıştır. 51 insan izolatı 13 değişik

plazmid profili göstermiştir. 21 tavuk izolatından 5 değişik plazmid profili elde edilmiştir. Diğer yandan, 10 yumurta izolatı da 6 değişik plazmid profili göstermiştir. Bursa ve Ankara yörelerinden elde edilen izolatlardan, insan, tavuk ve yumurta kökenli izolatların plazmit profilleri büyük benzerlik göstermiştir Çalışmalarımız sonucu izolatlar arasında 54 kb lik yüksek molekül ağırlıklı plazmidin insan, tavuk ve yumurta izolatlarında sırasıyla %94.1, %90.5 ve %100 oranlarında içerildiği gösterilmiştir. İzolatların değişik bakteriyofajlara karşı olan duyarlılık sonuçları faj tipi 4'ün (PT4) insan ve yumurta kökenli izolatlarda baskın olduğunu göstermesine karşın, tavuk izolatlarında ise faj tipi 6'nın (PT6) baskın olduğu ortaya çıkmıştır. Tüm izolatların genelde PT1, PT4, PT6 ve PT7 faj tiplerine ait olması insan, tavuk ve yumurta kökenli *S. enteritidis* izolatları arasındaki epidemiyolojik bağlantıyı göstermiştir. Toplam 82 *S. enteritidis* izolatının lipopolisakkarit (LPS) tabakaları proteinase K digestion ve SDS-PAGE yöntemleri kullanılarak incelenmiştir. Buna göre tavuk izolatlarının yüzde 24'ü, yumurta izolatlarının yüzde 40'ı ve insan izolatlarının %80'i düzgün (smooth) tip LPS profili göstermesine karşın, tavuk izolatlarının %76'sı, yumurta izolatlarının %60'ı ve insan izolatlarının %20'si düzgün olmayan (rough) tip LPS profili göstermiştir. İzolatların dış hücre zarı proteinleri, sarkosyl ekstraksiyon ve SDS-PAGE yöntemleri incelenmiştir. İzolatların tiplendirilmesi her bir izolat için proteinlerin sayısı ve molekül ağırlıkları dikkate alınarak yapılmış olup, tiplendirme sonucu dört değişik protein profili elde edilmiş, sırasıyla TipI, TipII, TipIII ve TipIV şeklinde adlandırılmıştır. Buna göre, insan izolatlarının %69'u, tavuk izolatlarının %38'i ve yumurta izolatlarının tamamı TipII profili göstermiştir. Diğer yandan 21 tavuk izolatının %52'sinin TipIII profile sahip olduğu bulunmuştur. Elde edilen antibiyotik dirençlilik, plazmit, faj tipi, lipopolisakkarit ve dış hücre zarı protein profil sonuçları *S. enteritidis* olmayan diğer 20 enterik bakteriden elde edilen sonuçlar ile karşılaştırılmıştır.

Anahtar kelimeler: *S. enteritidis*, Epidemiyoloji, Antibiyotik dirençlilik tiplendirmesi, Plazmit profil tiplendirmesi, Faj tiplendirmesi, Lipopolisakkarit profili, dış hücre zarı protein profili, Temel öğeler analizi

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S. enteritidis	:	*Salmonella enteritidis*
S. typhimurium	:	*Salmonella typhimurium*
S. typhi	:	*Salmonella typhi*
S. pullorum	:	*Salmonella pullorum*
S. gallinarum	:	*Salmonella gallinarum*
E. coli	:	*Escherichia coli*
PT	:	Phage Type
PP	:	Plasmid Profile
OMP	:	Outer Membrane Protein
MOMP	:	Major Outer Membrane Proteins
LPS	:	Lipopolysaccharide
KDa	:	Kilodalton
LB	:	Luria Broth
Kb	:	Kilobase
bp	:	Base Pair
Mda	:	Mega Dalton
SDS-PAGE	:	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

CHAPTER 1

INTRODUCTION

Bacterial foodborne illness is the number one food safety problem in the whole world, and cases caused by *Salmonella* bacteria are among the most common. The number of *Salmonella* infections reported has increased some 50 percent over the past decade, and many of these infections can be traced to raw or undercooked red meat and poultry. While not ordiranily life threatining, a *Salmonella* infection can be very serious especially in infants, the elderly, and people with immune system disorders. It is not surprising, therefore, that food industry, government, and university researchers are always on the lookout for new ways of reducing *Salmonella* in other foods.

Salmonella is a rod-shaped, motile bacterium - nonmotile exceptions *S. gallinarum* and *S. pullorum* -, non-sporeforming and Gram-negative (Fig. 1.1). There is widespread occurrence in animals, especially in poultry and swine.

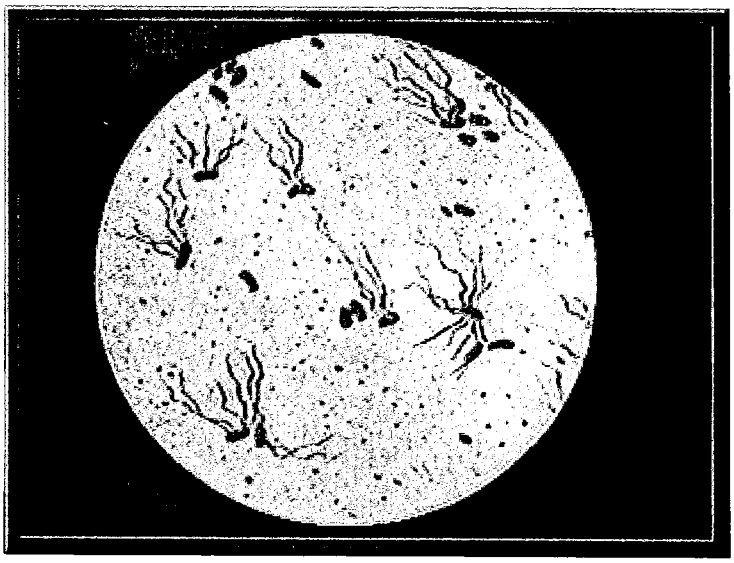


Figure 1.1. *Salmonella typhi* (from Tortora *et al.*, 1995)

Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces, raw meats, raw poultry, and raw seafoods. Transmission of this microbe is usually through uncooked meats and eggs. Chickens are major reservoir of *Salmonella*, which explains its ubiquitous presence in poultry products. Ingesting foods contaminated with significant amounts of *Salmonella* can cause intestinal infection which can eventually lead to diarrhea, vomiting, chills, and a really big headache.

The 2200 known species of *Salmonellae* are classified according to their surface antigens. The capsular properties of this Gram-negative rod can cause serious complications in immunosuppressed individuals such as HIV/AIDS patients. *S. thyphimurium* and *S. enteritidis* are two leading causes of salmonellosis (imflammation of the intestine caused by *Salmonella*). While most *Salmonella* are carried by animals, *S. typhi* is unique because it is only carried by humans.

Salmonella enteritidis, now established as an etiological agent of human gastroenteritis, is recognized worldwide as one of the most frequently isolated of the *Salmonella* serotypes causing food-borne disease outbreaks (Hadfield *et al.*, 1985; Lin *et al.*, 1988; Threlfall *et al.*, 1989; Rivera *et al.*, 1991). In the USA, UK and Spain, there was a 6-fold increase in reported isolations of *S. enteritidis* between 1973 and 1991 (Lin *et al.*, 1988; St Louis *et al.*, 1988; Echeita & Usera 1989; Cowden *et al.*, 1989; Dorn *et al.*, 1992). In England, *S. enteritidis* has caused significant economic losses due to condemnations of infected layer flocks (Lois *et al.*, 1994). In these European countries and the United States, the drastic increase in human salmonellosis from *S. enteritidis* has been the result of the consumption of contaminated eggs. Salmonellosis can also occur when consuming *S. enteritidis* contaminated egg products, poultry, or other meat products. It has been suggested that *S. enteritidis* contaminated eggs are the result of transovarian transmission, rather than fecal contamination of the shells (O'Brien *et al.*, 1982).

1.1. Pathogenesis

Salmonellosis includes several syndromes (gastroenteritis, enteric fevers, septicemia, focal infections, and an asymptomatic carrier state) (Fig. 1.2). Particular serovars show a strong propensity to produce a particular syndrome (*S typhi*, *S paratyphi*-A, and *S schottmuelleri* produce enteric fever; *S choleraesuis* produces septicemia or focal infections; *S typhimurium* and *S enteritidis* produce gastroenteritis); however, on occasion, any serotype can produce any of the syndromes.

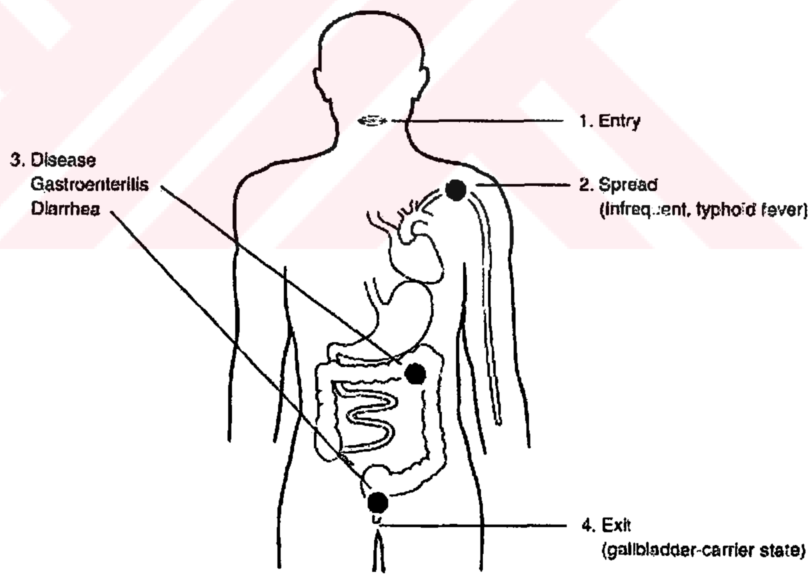


Figure 1.2. Pathogenesis of salmonellosis (from Gianella, 2000)

Most non-typhoidal salmonellae enter the body when contaminated food is ingested (Fig. 1.3). Person-to-person spread of salmonellae also occurs. To be fully pathogenic, salmonella must possess a variety of attributes called virulence factors. These include (1) the ability to invade cells, (2) a complete lipopolysaccharide coat, (3) the ability to replicate intracellularly, and (4) possibly the elaboration of toxin(s). After ingestion, the organisms colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles. The mechanism by which salmonellae invade the epithelium is partially understood and involves an initial binding to specific receptors on the epithelial cell surface followed by invasion. Invasion occurs by the organism inducing the enterocyte membrane to undergo "ruffling" and thereby to stimulate pinocytosis of the organisms (Fig. 1.4). Invasion is dependent on rearrangement of the cell cytoskeleton and probably involves increases in cellular inositol phosphate and calcium. Attachment and invasion are under distinct genetic control and involve multiple genes in both chromosomes and plasmids.

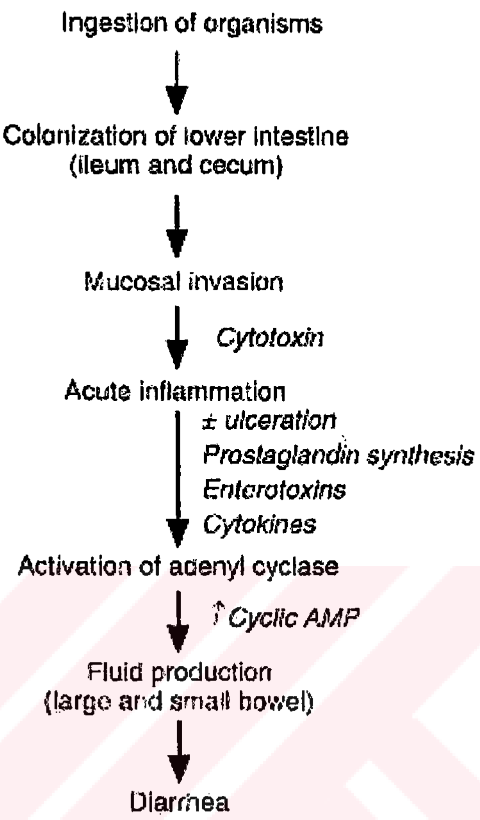


Figure 1.3. Scheme of the pathogenesis of *Salmonella enterocolitis* and diarrhea (from Gianella, 2000).

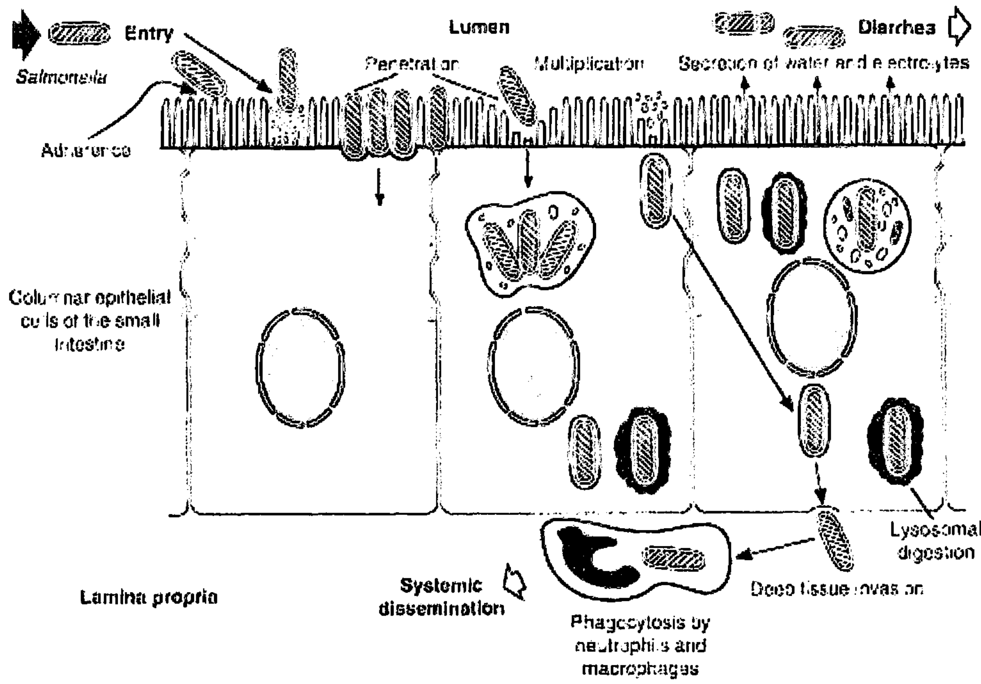


Figure 1.4. Invasion of intestinal mucosa by *Salmonella* (from Gianella, 2000)

After invading the epithelium, the organisms multiply intracellularly and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation; they are taken up by the reticuloendothelial cells. The reticuloendothelial system confines and controls spread of the organism. However, depending on the serotype and the effectiveness of the host defenses against that serotype, some organisms may infect the liver, spleen, gallbladder, bones, meninges, and other organs (Fig. 1.2). Fortunately, most serovars are killed promptly in extraintestinal sites, and the most common human *Salmonella* infection, gastroenteritis, remains confined to the intestine.

After invading the intestine, most salmonellae induce an acute inflammatory response, which can cause ulceration. They may elaborate cytotoxins that inhibit protein synthesis. Whether these cytotoxins contribute to the inflammatory response or to ulceration is not known. However, invasion of the mucosa causes the epithelial cells to synthesize and release various proinflammatory cytokines, including: IL-1, IL-6, IL-8, TNF-2, IFN-U, MCP-1, and GM-CSF. These evoke an acute inflammatory response and may also be responsible for damage to the intestine. Because of the intestinal inflammatory reaction, symptoms of inflammation such as fever, chills, abdominal pain, leukocytosis, and diarrhea are common. The stools may contain polymorphonuclear leukocytes, blood, and mucus.

Much is now known about the mechanisms of Salmonella gastroenteritis and diarrhea. Figures 1.3 and 1.4 summarize the pathogenesis *of Salmonella enterocolitis* and diarrhea. Only strains that penetrate the intestinal mucosa are associated with the appearance of an acute inflammatory reaction and diarrhea (Fig. 1.5); the diarrhea is due to secretion of fluid and electrolytes by the small and large intestines. The mechanisms of secretion are unclear, but the secretion is not merely a manifestation of tissue destruction and ulceration. Salmonella penetrate the intestinal epithelial cells but, unlike *Shigella* and invasive *E. coli*, do not escape from the phagosome. Thus, the extent of intercellular spread and ulceration of the epithelium is minimal. *Salmonella* escape from the basal side of epithelial cells into the lamina propria. Systemic spread of the organisms can occur, giving rise to enteric fever. Invasion of the intestinal mucosa is followed by activation of mucosal adenylate cyclase; the resultant increase in cyclic AMP induces secretion. The mechanism by which adenylate cyclase is stimulated is not understood; it may involve local production of prostaglandins or other components of the inflammatory reaction. In addition, *Salmonella* strains elaborate one or more enterotoxin-like substances which may stimulate intestinal secretion. However, the precise role of these toxins in the pathogenesis of *Salmonella enterocolitis* and diarrhea has not been established.

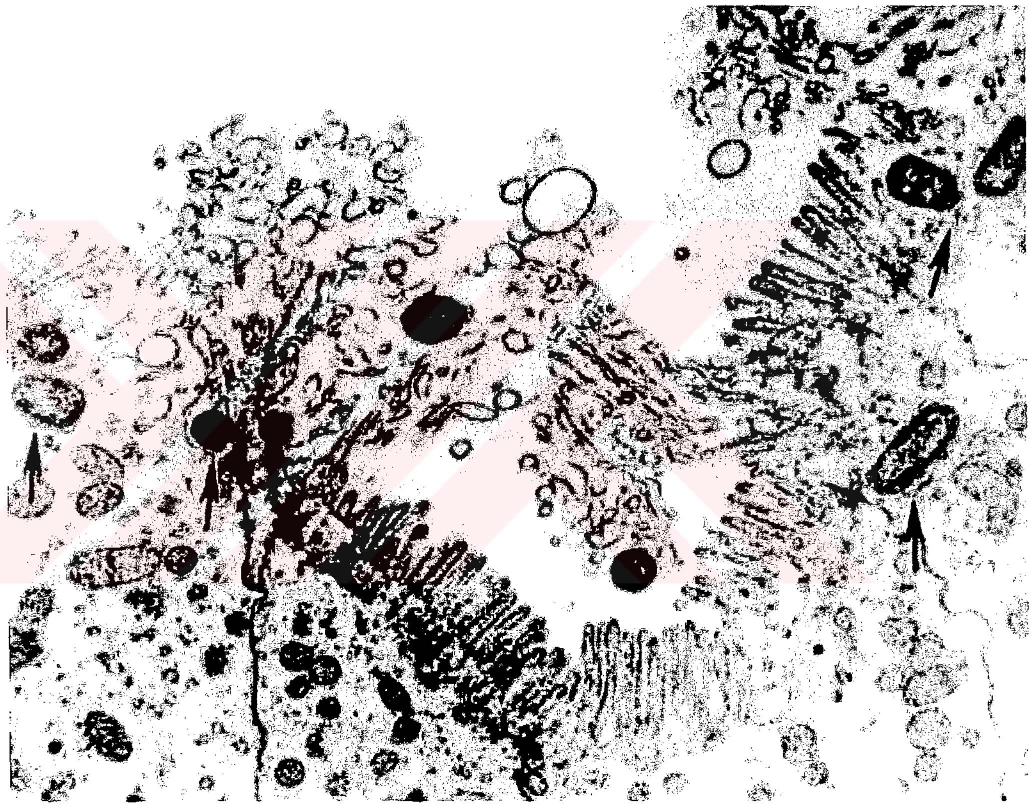


Figure 1.5. Electron photomicrograph demonstrating invasion of guinea pig ileal epithelial cells by *Salmonella typhimurium*. Arrows point to invading Salmonella organisms. (from Gianella, 2000).

1.2. Host Defenses

Various host defenses are important in resisting intestinal colonization and invasion by *Salmonella* (Table 1.1). Normal gastric acidity ($pH < 3.5$) is lethal to salmonellae. In healthy individuals, the number of ingested salmonellae is reduced in the stomach, so that fewer or no organisms enter the intestine. Normal small intestinal motility also protects the bowel by sweeping ingested salmonellae through quickly. The normal intestinal microflora protects against salmonellae, probably through anaerobes, which liberate short-chain fatty acids that are thought to be toxic to salmonellae. Alteration of the anaerobic intestinal flora by antibiotics renders the host more susceptible to salmonellosis. Secretory or mucosal antibodies also protect the intestine against salmonellae. Animal strains genetically resistant to intestinal invasion by salmonellae have been described. When these host defenses are absent or blunted, the host becomes more susceptible to salmonellosis; factors that render the host more susceptible to salmonellosis are listed in Table 1.2. For example, in AIDS, *Salmonella* infection is common, frequently persistent and bacteremic, and often resistant to even prolonged antibiotic treatment. Relapses are common. The role of host defenses in salmonellosis is extremely important, and much remains to be learned.

Table 1.1. Host defenses against *Salmonellae* (from Gianella, 2000)

Host Defense	Examples of Factors
Gastric Factors	Gastric acidity
	Rate of gastric emptying
Intestinal Factors	Intestinal motility
	Normal intestinal flora
	Mucus
	Secretory antibodies
	Genetic resistance to invasion
Nonspecific and Other Possible Factors	Nutritional state
	Lactoferrin
	Gut reticuloendothelial cells
	Lysozyme

Table 1.2. Factors increasing susceptibility to salmonellosis (from Gianella, 2000)

Location or Factor	Specific Condition
Stomach	Achlorhydria
	Gastric Surgery
Intestine	Antibiotic administration
	Gastrointestinal surgery
	? Idiopathic inflammatory bowel disease
Hemolytic Anemias	Especially sickle cell anemia and other hemoglobinopathies
Impaired Systemic Immunity	Carcinomatosis, leukemias, lymphomas
	Diabetes mellitus, Immunosuppressive drugs, acquired immunodeficiency syndrome (AIDS),etc

1.3. Epidemiology

Contaminated food is the major mode of transmission for salmonellae because salmonellosis is a zoonosis and has an enormous animal reservoir. The most common animal reservoirs are chickens, turkeys, pigs, and cows; dozens of other domestic and wild animals also harbor these organisms. Because of the ability of salmonellae to survive in meats and animal products that are not thoroughly cooked, animal products are the main vehicle of transmission. The magnitude of the problem is demonstrated by the following recent yields of salmonellae: 41% of turkeys examined in California, 50% of chickens cultured in Massachusetts, and 21% of commercial frozen egg whites examined in Spokane, WA (Gianella, 2000).

On the other hand, the epidemiology of typhoid fever and other enteric fevers primarily involves person-to-person spread because these organisms lack a significant animal reservoir. Contamination with human feces is the major mode of spread, and the usual vehicle is contaminated water. Occasionally, contaminated food (usually handled by an individual who harbors *S. typhi*) may be the vehicle. Plasmid DNA fingerprinting and bacterial phage lysotyping of *Salmonella* isolates are powerful epidemiologic tools for studying outbreaks of salmonellosis and tracing the spread of the organisms in the environment (Gianella, 2000).

In typhoid fever and non-typhoidal salmonellosis, two other factors have epidemiologic significance. First, an asymptomatic human carrier state exists for the agents of either form of the disease. Approximately 3% of persons infected with *S. typhi* and 0.1% of those infected with non-typhoidal salmonella become chronic carriers. The carrier state may last from many weeks to years. Thus, human as well as animal reservoirs exist. Interestingly, children rarely become chronic typhoid carriers. Second, use of antibiotics in animal feeds and indiscriminant use of antibiotics in humans increase antibiotic resistance in salmonella by promoting transfer of R factors.

Salmonellosis is a major public health problem because of its large and varied animal reservoir, the existence of human and animal carrier states, and the lack of a concerted nationwide program to control salmonella .

1.4. Diagnosis

The diagnosis of salmonellosis requires bacteriologic isolation of the organisms from appropriate clinical specimens. Laboratory identification of the genus Salmonella is done by biochemical tests; the serologic type is confirmed by serologic testing. Feces, blood, or other specimens should be plated on several nonselective and selective agar media (blood, MacConkey, eosin-methylene blue, bismuth sulfite, *Salmonella-Shigella*, and brilliant green agars) as well as into enrichment broth such as selenite or tetrathionate. Any growth in enrichment broth is subsequently subcultured onto the various agars. The biochemical reactions of suspicious colonies are then determined on triple sugar iron agar and lysine-iron agar, and a presumptive identification is made. Biochemical identification of salmonellae has been simplified by systems that permit the rapid testing of 10-20 different biochemical parameters simultaneously. The presumptive biochemical identification of Salmonella then can be confirmed by antigenic analysis of O and H antigens using polyvalent and specific antisera. Fortunately, approximately 95% of all clinical isolates can be identified with the available group A-E typing antisera. Salmonella isolates then should be sent to a central or reference laboratory for more comprehensive serologic testing and confirmation (Gianella, 2000).

1.5. Control

Salmonellae are difficult to eradicate from the environment. However, because the major reservoir for human infection is poultry and livestock, reducing the number of salmonellae harbored in these animals would significantly reduce human exposure. In Denmark, for example, all animal feeds are treated to kill salmonellae before distribution, resulting in a marked reduction in salmonellosis (Gianella, 2000). Other helpful measures include changing animal slaughtering practices to reduce cross-contamination of animal carcasses; protecting processed foods from contamination; providing training in hygienic practices for all food-handling personnel in slaughterhouses, food processing plants, and restaurants; cooking and refrigerating foods adequately in food processing plants, restaurants, and homes; and expanding of governmental enteric disease surveillance programs.

Recently, The U.S. Department of Agriculture has approved the radiation of poultry to reduce contamination by pathogenic bacteria, e.g. Salmonella and Campylobacter. Unfortunately, radiation pasteurization has not yet been widely accepted in the U.S. Adoption and implementation of this technology would greatly reduce the magnitude of the salmonella problem (Gianella, 2000).

Vaccines are available for typhoid fever and are partially effective, especially in children. No vaccines are available for non-typhoidal salmonellosis. Continued research in this area and increased understanding of the mechanisms of immunity to enteric infections are of great importance (Gianella, 2000).

General salmonellosis treatment measures include replacing fluid loss by oral and intravenous routes, and controlling pain, nausea, and vomiting. Specific therapy consists of antibiotic administration. Typhoid fever and enteric fevers should be treated with antibiotics. Antibiotic therapy of non-typhoidal salmonellosis should be reserved for the septicemic, enteric fever, and focal infection syndromes. Antibiotics are not recommended for uncomplicated Salmonella gastroenteritis because they do not shorten the illness and they significantly prolong the fecal excretion of the organisms and increase the number of antibiotic-resistant strains (Gianella, 2000).

1.6. *Salmonella* Transmission Cycles

A patient with salmonellosis may excrete the organism for many weeks and in very large numbers. *Salmonella* from infected people, but also from slaughterhouses and butchers shops, and of course from non-symptomatic animal carriers, both domesticated and free-living, are brought into the environment. It has been shown that *Salmonella* can occur in large quantities in municipal sewage systems, and that from these surface waters may be polluted, since purification of sewage water often does not lead to the reduction of *Salmonella* (Oosterom *et al.*, 1980). From these waters, but also from dung and slurry reservoirs, insects, rodents and birds may be contaminated with *Salmonella*, and these animals can bring

Salmonella back to farm animals, animal feeds, foodstuffs and to man himself. It has been demonstrated by extensive studies, involving serotyping and phage-typing of isolates, that *Salmonella* transmission cycles are established in this way (Edel *et al.*, 1978). These cycles show the capacity to regenerate themselves, because in many stages, particularly in the gut of warm-blooded animals and man, multiplication of *Salmonella* occurs (Oosterom *et al.*, 1980). Even outside the body, if temperature and humidity are favourable, *Salmonella* can propagate. Reduction of *Salmonella* may occur in other stages, in general during hygienic slaughter and during the final preparation of meat. But this is not sufficient to interrupt the cycle for longer periods of time. The basic infection cycle is represented in Figure 1.6.

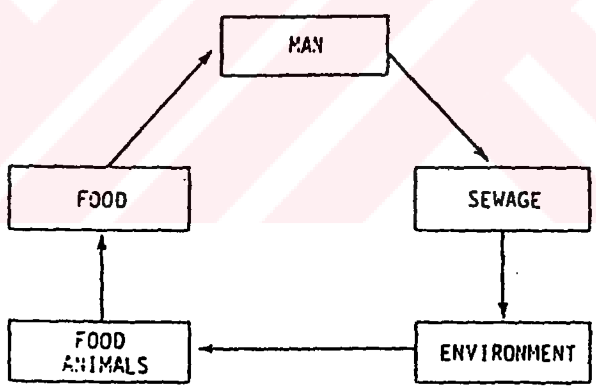


Figure 1.6. Major cycle of *Salmonella* (from Oosterom, 1991)

The cycle discussed above is of course a gross simplification of reality. Many transmission pathways do exist, and most of them are part of one or more epidemiological cycles. There is for instance transmission of *Salmonella* from slaughter offals to pet dogs and possibly from these to children. In some cases slaughter offals, sewage sludge or even chicken droppings are used as feed components for cattle and pigs. *Salmonella* may also enter the home by way of vegetables harvested from soil fertilised with pig slurry, or by way of fish caught in polluted water. Person-to-person sepread of *Salmonella* may occur as well. When all knowledge about possible transmission pathways of *Salmonella* is collected, the complex, but instructive diagram of Figure 1.7 appears.

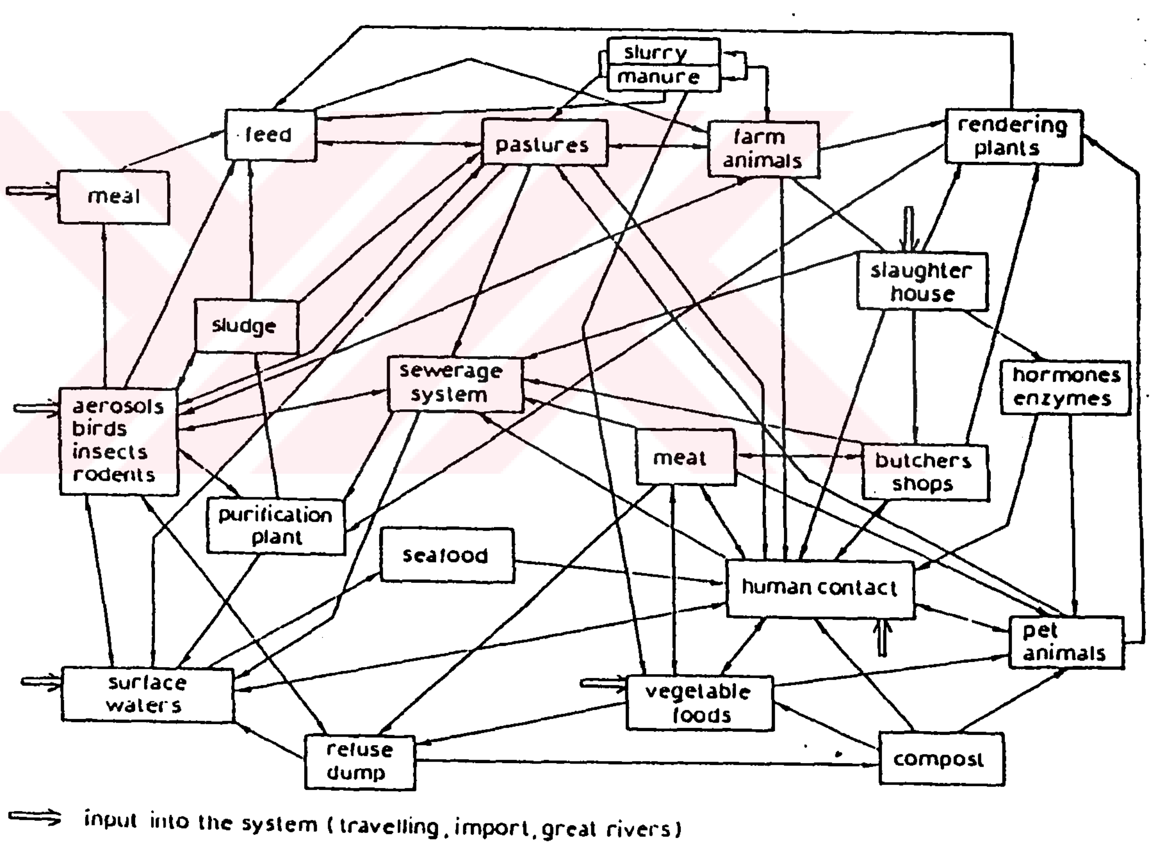


Figure 1.7. Possible pathways of *Salmonella* contamination (from Oosterom, 1991)

It is clear that many transmission routes exist, and that man can be reached by many. In order to disrupt this constant flow towards man, many epidemiological measures should be carried out simultaneously. There is no point in taking action in one field and neglecting others. So for prevention, a close cooperation between farmers, feed suppliers, slaughterhouses, butchers' shops, personnel in kitchens and people involved in waste disposal is needed to fight the problem effectively.

1.7. *Salmonella enteritidis* in Poultry and Eggs

For many years it has been discussed whether *Salmonella* can be found inside chicken eggs, like in duck eggs, but it has never been proved. Now it has sufficiently been demonstrated that *S. enteritidis* can cause general infection in chickens, which can lead to infection of the ovaries and therewith of the internal material of the eggs (O'Brian, 1988; Hopper and Mawer, 1988). This means that external cleaning and disinfection of eggs is no longer enough, and the same is true for minimal cooking or baking. The organism can even survive in scrambled eggs which are heated for longer periods of time (Lin *et al.*, 1988). Other products drived from eggs, such as mayonnaise, tartare sauce, egg-nog, milk shakes, mousse and ice cream are likewise implicated in outbreaks of *S. enteritidis* in the general population (Anonymous, 1988).

It is not generally realised that this *Salmonella* serotype also causes general infection in the chicken, and that colonisation of the gut may remain as important a factor as with other *Salmonella* serotypes. This means that among the feacal *Salmonella* on poultry meat *S. enteritidis* can also be present, and, like in other serotypes, can cause foodborne infection. A further impliction is that many human infections with *S. enteritidis* do not necessarily originate from infected eggs, but may be caused by contaminated poultry meat.

1.8. Biochemical and Serological Identification

The association of *S. enteritidis* with disease in humans and other animals has increased the importance of epidemiological studies. Detailed strain identification is essential for useful investigations of *Salmonella* outbreaks. Epidemiological investigations have traditianolly relied on biochemical and serological methods for the primary identification of strains. Recognition of a number of phenotypic properties has been used for their subsequent differentiation. In contrast, modern typing methods are based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA. Phenotyping methods include antimicrobial susceptibility testing (Bichler and Nagaraja, 1989; O'Brien *et al.*, 1982), biotyping , colicin typing , and phage typing (Holmberg *et al.*, 1984). These methods have been supplemented by genetic characterization, such as DNA fingerprinting (Kim and Nagaraja 1988) and plasmid profile analysis (Helmuth *et al.*, 1985).

1.8.1. Biochemical Identification

Enzymatic activities are widely used to differentiate among bacteria. Even closely related bacteria can usually be separated into distinct species by subjecting them to biochemical tests, such as their ability to ferment an assortment of selected carbohydrates. The time needed to identify bacteria can be reduced considerably by the use of selective and differential media or by rapid identification methods. Rapid identification tools are manufactured for groups of medically important bacteria, such as enterics. Such tools are designed to perform several biochemical tests simultaneously and can identify bacteria within 4 to 24 hours (Threlfall and Frost, 1990).

The serotype Enteritidis (*S. enteritidis*) belongs to a single species, *Salmonella enterica* (Le Minor, 1984). Subdivision into subspecies utilizes biochemical reactions of strains with dulcitol, lactose, o-nitrophenol-β-D-galactopyronoside (ONPG), salicin, D-tartrate, mucate, malonate, gelatinase, sorbitol and potassium cyanide (KCN). Seven subspecies have been recognized I, II, IIIa and IIIb, IV, V and VI (Le Minor, 1988). Before 1966, serovars of subspecies II, IV, V and VI were named but have subsequently been referred to by their antigenic formulae. Members of subspecies IIIa are referred to as *Salmonella arizonae* and subspecies IIIb as *Salmonella diarizonae*, are further defined by their antigenic formula.

1.8.2. Serological Identification

Microorganisms are antigenic; that is, microorganisms that enter an animal's body stimulate it to form antibodies. *Antisera* (singular: antiserum) are commercially available solutions of such antibodies used in the identification of many medically important microorganisms. If an unknown bacterium is isolated from a patient, it can be tested against known antisera and often identified quickly.

In a procedure called a **slide agglutination test**, samples of an unknown bacterium are placed in a drop of saline on each several slides. Then a different known antiserum is added to each sample. The bacteria agglutinate (clump) when mixed with antibodies that were produced in response to that species or strain of bacterium; a positive test is indicated by the presence of agglutination.

Serological techniques can differentiate not only among microbial species but also among strains within species.

Enzyme-linked immunosorbent assays (ELISAs) are now widely used because they are fast and can be read by a computer scanner. In a direct ELISA, known antibodies are placed in the wells of a microplate, and an unknown type of bacteria is added to each well. A reaction between the known antibodies and the bacteria provides identification of the bacteria.

Another serological test, the **Western blot,** is used to identify bacterial antigens in a patient's serum. (1) Proteins (including bacterial proteins) in the patient's serum are separated by a process called electrophoresis. (2) The proteins are then transferred to the filter by blotting. (3) Next, antibodies tagged with a dye are washed over the filter. If the specific antigen is present in the serum, the antibodies will combine with it and will be visible as a colored band on the filter.

Strains of *Salmonella* spp. are classified into serovars on the basis of extensive diversity of the lipopolysaccharide (O) antigens and the flagellar protein

(H) antigens, in accordance with the scheme instituted by White (1926) and extended and elaborated by Kauffmann (1972; 1978). Over 2000 antigenically distinct serovars have now been recognized (Le Minor, 1984; Ewing, 1986; Rowe and Hall, 1989).

The most common serovars which cause infections in humans and food animals belong to subspecies I. 1289 named serovars of subspecies I have been defined and about 99% of *Salmonella* strains from humans belong to this subspecies (Le Minor, 1988). In contrast, serovars belonging to subspecies II, III, IV, V and VI are common in reptiles but are rarely isolated from humans. Strains of *Salmonella arizonae* have been found in sheep in the United Kingdom, but cause only a few human infections each year (Hall and Raw, 1980).

1.9. Subdivision within Serovar-phenotypic Characterization

Although over 1000 serovars of subspecies Threlfall and Frost (1990) have been identified, the majority are rarely isolated from humans in England and Wales and are consequently not of epidemiological importance. In contrast, *S. typhimurium, S. enteritidis* and *S. virchow* cause over 60% of all *Salmonella* infections each year (Palmer and Raw, 1986). Subdivision of the most common serovars, and other serovars of particular clinical importance is often required to facilitate in investigations into outbreaks.

A variety of phenotypic characteristics has been used both independently and in combination for serovar subdivision. Phenotypic methods of typing have been supplemented by genetic characterization of plasmids, recognition of differences in outer membrane protein profiles and analysis of lipopolysaccharide content.

1.10. Phage Typing

Phage typing is a test for determining which phages a bacterium is susceptible to. One bacterial strain might be susceptible to two different phages, whereas another strain of the same species might be susceptible to those two phages plus a third phage.

The sources of food-associated infections can be traced by phage typing. One version of this procedure starts with a plate totally covered with bacteria growing on agar. A drop of each different phage type to be used in the test is then placed on the bacteria. Wherever the phages are able to infect and lyse the bacterial cells, clearings in the bacterial growth (called plaques) appear (Figure 1.8).

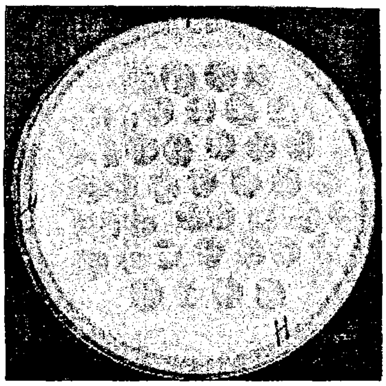


Figure 1.8. Phage typing of a strain of *Salmonella typhi* (from Tortora *et al.*, 1995)

Such test might show, for instance, that bacteria isolated from a surgical wound have the same pattern of phage sensitivity as those isolated from the operating surgeon or surgical nurses. This establishes that the surgeon or a nurse is the source of infection.

The first phage typing scheme based on the principal of phage adaptation and used on an international scale was that developed in 1938 for the differentiation of *S. typhi* (Craigie and Yen, 1938a, b). This scheme uses progressive adaptations of Vi-phage II, a phage specific for the Vi (capsular) antigen of *S. typhi* by varying the host strain (Felix and Pitt, 1934). These adaptations are used at the routine test dilution, the highest dilution that produces confluent lysis on the homologous bacterial phage type (Anderson, 1959). Eleven phage types were initially identified. In 1947, the method of *S. typhi* Vi phage typing was standardized (Craigie and Felix, 1947) and by 1986, with further adaptations of Vi-phage II, a further 95 types had been defined and internationally recognized, bringing the total number of Vi phage types to 106 (Edelman and Levine, 1986).

As mentioned above, the phage typing scheme for *S. typhi* depends on adaptations of one phage, Vi-phage II. In contrast, phage typing schemes for other *Salmonella* serovars depend to only a limited extent on phage adaptability and are, for the most part, based on the patterns of lysis produced by serologically distinct phages isolated from a variety of sources. Published phage typing schemes , other than that for *S. typhi*, which are in routine use in Britain include those for *S. paratyphi* B (Felix and Callow, 1943, 1951), *S. typhimurium* (Callow, 1959;

Anderson, 1964), *S. hadar* (de Sa *et al*., 1980), *S. virchow* (Chambers *et al*., 1987) and *S. enteritidis* (Ward *et al*., 1987). Over 200 phage types of *S. typhimurium* have been designated (Anderson *et al*., 1977) and a new phage typing scheme has been developed in India for typing untypable.strains of *S. typhimurium* which are common in the Indian sub-continent (Adlaka *et al*., 1986). On the other hand, 27 phage types were described by Ward and her friends in 1987.

1.11. *Salmonella* Identification, Typing and Fingerprinting

1.11.1. Biotyping

A typing scheme based on fermentation of nine substrates, which was developed in 1937, recognized 21 biotypes of *S. typimurium* (Kristensen *et al*., 1937). More recently, an extended two-tier scheme based on the original scheme of Kristensen *et al*., (1937) distinguishes 32 possible primary biotypes. In this extended scheme, subtypes within the primary types are distinguished by 10 further tests, including motility and presence of haemaggulitinating fimbriae. Twenty-four primary and 184 full biotypes have been identified (Duguid *et al*., 1975). Biotyping is more laborious and possibly less discriminatory than phage typing for *S. typhimurium*, but when the two methods are combined the degree of strain discrimination may be enhanced (Anderson *et al*., 1978; Barker *et al*., 1980; Barker and Old, 1980).

Biotyping has also been used to subdivide *S. paratyphi* B. Thirteen primary and 56 full biotypes have been recognized and it has been suggested that biotyping may be more discriminatory than phage typing for this serovar (Barker *et al*., 1988).

1.11.2. Colicin Typing

Colicinogeny in *Salmonella* is a property encoded by both conjugative and non-conjugative plasmids. The most common colicins produced by strain of *S. typhimurium* are encoded by conjugative plasmids of the I group-Col Ia and Col Ib (Fredericq, 1952; Hamon and Peron, 1966; Anderson *et al*., 1973). Because of the conjugative nature of these plasmids, the ability of starins to produce group I colicins cannot reliably be used as a typing system. Colicins of the E group, in particular colicins E1 and E2, but also colicins K, B, M and S4 have also been identified in *Salmonella* (Barker, 1980).

1.11.3. Drug Resistance (Antibiogram) Typing

Antibiotic resistance in *Salmonella* strains, in general, encoded by plasmids which have been acquired as a consequence of antibiotic pressure in human and veterinary medicine (Anderson, 1968; Threlfall *et al*., 1978a; Richmond and Linton, 1980). In turn, the majority of resistance plasmids have acquired their resistance genes by transposition, either from other plasmids in the strain, from the chromosome, or from plasmids carried by other bacterial strains in the host organism. Resistance may also have resulted from the spontaneous mutation of a chromosomal gene, again in response to selective pressure. Because of the fluidity of resistance plasmids and transposons, antibiotic resistance patterns *per se* cannot be regarded as a satisfactory primary method for discrimination within serovars. Nevertheless, antibiotic resistance typing can be used in conjunction with serotyping, biotyping, phage typing and genetic characterization of resistance plasmids for epidemiological purposes.

1.11.4. Plasmid Characterization

Plasmids are covalently-closed, circular, double-stranded extrachromosomal molecules of DNA which replicate independently of the bacterial chromosome. They encode a variety of functions which, in theory, are not essential for the survival of the cell in non-selective environments. Some plasmids code for properties such as antibiotic resistance and heavy metal resistance, which confer an obvious selective advantage on the host strain. Other plasmids code for properties which contribute to the virulence of the organism. However, phenotypic characteristics have not yet been identified for many of the plasmids found in enterobacterial strains.

Plasmids are genetically characterized by examination of properties such as transfer kinetics, fertility inhibition, bacteriophage restriction and incompatibility (Anderson and Threlfall, 1974; Frost *et al.*, 1983). Of these properties, incompatibility grouping, which is based on the inability of isogenic plasmids to co-exist in the same strain, has been used to develop an internationally recognized scheme of plasmid classification. Over 30 plasmid incompatibilty groups have now been defined (Jacob *et al.*, 1977).

1.12. Application of Phenotypic Typing Methods to Epidemiological Investigations

Identification of serovar without further differentiation enables observation of overall changes in the prevalence of particular serovars (Palmer and Rowe, 1986). For example, an upsurge in the incidence of *S. agona* in Britain in the mid-1970s was caused by the introduction of this serovar in imported fishmeal used as animal feed.

A sudden rise in the isolation rates of *S. hadar* in 1979 and 1980 was followed by its equally rapid decline over the next three years. Since 1981, *S. virchow* has increased to such an extent that by 1986, this serovar had become the third most common serovar in humans. More recently, serotyping has demonstrated that *S. enteritidis* displaced *S. typhimurium* in 1988 as the most common serovar causing food-poisoning in humans in England (Anon, 1989).

Identification of the serovar alone may also be sufficient in outbreaks caused by uncommon serovars. For example, an increase in cases of *S. napoli* amongst children in Britain was shown to have been caused by the consumption of chocolate bars imported from abroad (Gill *et al.*, 1983). Because of the rarity of this serovar, further discrimination was unnecessary. Likewise, apparently unrelated cases of *S. ealing* in infants in Britain in late 1985 and early 1986 were linked to the consumption of factory-dried milk powder (Rowe *et al.*, 1987). This outbreak was contained by withdrawal of the contaminated product and closure of the factory.

Since the 1950s, phage typing has been the method of choice in the reference laboratory for differentiating within serovars. The technique is rapid and provides a reproducable and highly discriminatory method of subdivision, using reagents which are stable and relatively inexpensive to produce. Phage typing has been particularly important in support of epidemiological investigations of *S. typhi* (Conn *et al.*, 1972; Anderson and Hobbs, 1973), *S. paratyphi* B (Anderson, 1964), *S. typhimurium* (Anderson, 1968; Threlfall *et al.*, 1978a, b; Mitchell *et al.*, 1989) and *S. hadar* (Rowe *et al.*, 1980). In 1988, phage typing demostrated that the recent upsurge of *S.*

enteritidis in humans in England and Wales was caused almost exclusively by strains belonging to phage type 4 (Anon, 1989; Char *et al.*, 1989a). Subsequent epidemiological investigations have identified this phage type in poultry (O'Brien, 1988) and shell eggs (Coyle *et al.*, 1989).

Although phage typing is essential for the primary subdivision of serovars, the method can prove insufficiently discriminatory in serovars in which a small number of phage types predominate. For example, from 1981 to 1986, 86% of strains of *S. enteritidis* isolated in Britain belonged to phage types 4 and 8 (Ward *et al.*, 1987); further discrimination within these phage types is obviously desirable. Likewise, further differentiation of common *S. typhimurium* phage types, in particular phage types 12 and 49 (Palmer and Rowe, 1986), may be required to assist epidemiological investigations.

Biotyping has been used to supplement phage typing for the subdivision of *Salmonella* serovars. Strains of *S. typhimurium* phage type 141 isolated from incidents in the Sheffield area in 1984 and 1985 were found to belong to biotype 31beg but previous strains isolated in different parts of the country belonged to unrelated biotypes (Old and Barker, 1989).

Colicin typing and antibiogram (resistance) typing cannot be used in isolation for serovar subdivision of *Salmonella* since, for the most part, both colicin production and drug resistance are encoded by conjugative or mobilizable plasmids. Furthermore, resistance to a particular antibiotic or antibiotics can be conferred by totally different plasmids, combining to produce the same phenotypic resistance pattern in unrelated strains. Nevertheless, colicin typing has been used to supplement phage typing and biotyping for the subdivision of strains of *S. typhimurium* belonging to phage type 141 (Old and Barker, 1989). Similarly, resistance typing used in combination with phage typing and plasmid characterization has provided a means of identifiying multiple drug-resistant clones of *S. typhimurium* belonging to phage types 29 (Anderson, 1968), 204 (Threlfall *et al.*, 1978a), 193 (Threlfall *et al.*, 1978b) and 204c (Threlfall *et al.*, 1980). Multiply resistant clones of the above phage types, initially identified by their distinctive resistance patterns, have spread in cattle in Britain in the 1960s (phage types 29), the late 1970s (phage types 204 and 193) and the 1980s (phage type 204c) and caused many infections in humans. Genetic characterization of the resistance plasmids carried by strains of *S. typhimurium* phage types 204, 193 and 204c has demonstrated that these phage types are closely related and that changes of phage type have been caused by the sequential acquisition of resistance plasmids as a result of selective pressure (Threlfall *et al.*, 1987a, b ; Threlfall *et al.*, 1980).

Resistance typing has also been the principal method of identifying clones of chloramphenicol resistant *S. typhi* belonging to different phage types which have caused major outbreaks in Mexico, India and Southeast Asia since the early 1970s. Genetic characterization of the chloramphenicol resistance plasmids has demostrated that, in all strains, resistance was encoded by plasmids belonging to the H1 incompatability group, indicating a degree of affinity of H1 plasmids for *S. typhi* (Anderson, 1975; Rowe and Threlfall, 1984).

1.13. Outer Membrane Protein

The cell envolope of the family Enterobacteriaceae consists of three layers: a cytoplasmic membrane, a peptidoglycan layer and, covalently attached to it, an outer membrane (DiRienzo *et al.*, 1978). The protein pattern of the outer membrane is dominated by a few proteins such as murein lipoprotein and classical porins.

Analysis of the outer membrane protein pattern with sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis revealed several so-called 'major' proteins (Ames *et al.*, 1974; Lugtenberg *et al.*, 1975). As the relative amounts of several of these proteins are dependent on the growth conditions (Van Alphen and Lugtenberg, 1977; Braun *et al.*, 1976; Braun and Krieger-Brauer, 1977; Lugtenberg *et al.*, 1976; Overbeeke and Lugtenberg, 1980), this term can be misleading. The outer membrane protein pattern of *Escherichia coli* K12 is characteristic in that usually three major bands are observed in the molecular weight range between 30000 and 42000. The nomenclature of these proteins (for instance, designated b,c and d by Lugtenberg and his coworkers 1975) was different in different laboratories (Bassford *et al.*, 1977; Henning and Haller, 1975; Uemura and Mizushima, 1975). Recently, a new nomenclature has been agreed in which the proteins are called after their structural genes (Reeves, 1979), i.e. *ompF*, *ompC* and *ompA*, respectively, for the proteins b,c and d (Overbeeke and Lugtenberg, 1980).

The proteins coded for by *ompF*, *ompC*, and *phoE* genes in *E. coli* and the homologous genes plus *omp*D in *S. typhimurium* are trimeric porins that produce relatively nonspecific pores or channels that allow the rapid passage of small hydrophilic molecules across the OM. Among these porins, the PhoE protein is unique in that it is produced only under conditions of phosphate starvation. Thus, in the usual culture media, only the OmpF and OmpC (and in *S. typhimurium* also OmpD) porins are produced, but the relative abundance of these porins is under an efficient regulation by environmental signals (Curtis III *et al.*, 1996).

When the OM-peptidoglycan complex is extracted with sodium dodecyl sulphate (SDS) at temperatures below 60°C, much of the porin is left behind in the insoluble fraction (Rosenbusch, 1974). Porins can be solubilized as undenatured, tightly associated trimers by including 1 M NaCl in the SDS solution. It requires heating in SDS at temperatures above 70°C to denature the porins and dissociate them into monomeric subunits (Curtis III *et al.*, 1996).

The OmpA protein, which plays a role in acceptor cells in F-pilus-mediated conjugation (Van Alphen *et al.*, 1977; Skurray *et al.*, 1974), is partially degraded when cell envelope preparations are incubated with trypsin, resulting in a major degradation product with a molecular weight of about 18000 (Henning *et al.*, 1973): The OmpF and OmpC proteins form tight complexes with peptidoglycan which resist incubations in 2% SDS at 60°C (Lugtenberg *et al.*, 1976; Rosenbusch, 1974).

Analysis of outer membrane protein (OMP) patterns has also been applied to the subdivision of *Salmonella* serovars. In contrast to findings with *Escherichia coli* K1 (Achtman *et al.*, 1983), major OMP profiles were homogeneous within a serovar in all of seven *Salmonella* serovars tested. Although small variations were observed in low molecular weight proteins, it was concluded that OMP patterns may not be very useful for epidemiological studies (Helmuth *et al.*,1985).

1.14. Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) analysis, using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a further method which has been used for the characterization of *Salmonella* strains. Typically, lipopolysaccharides of *Salmonella* separate polysaccharide chains of different lengths, to produce a 'ladder' pattern which varies between serovars (Hitchcock and Brown, 1983). Loss of the ability to synthesize LPS, however, has been correlated with conversion of *S. enteritidis* phage type 4 to phage type 7 (Chart *et al.*, 1989).

1.14.1. Mechanisms of Bacterial Pathogenicity: Endotoxins

Endotoxins are part of the outer cell wall of bacteria. Endotoxins are invariably associated with Gram-negative bacteria as constituents of the outer membrane of the cell wall. Although the term endotoxin is occasionally used to refer to any "cell-associated" bacterial toxin, it should be reserved for the lipopolysaccharide complex associated with the outer envelope of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens.

The biological activity of endotoxin is associated with the lipopolysaccharide (LPS) layer, as mentioned in the preceding section. Toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components. The cell wall antigens (O antigens) of Gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses in an animal. Because it activates complement by the alternative (properdin) pathway, it is often part of the pathology of Gram-negative bacterial infections.

The relationship of endotoxins to the bacterial cell surface is illustrated in Figure 1.9 below.

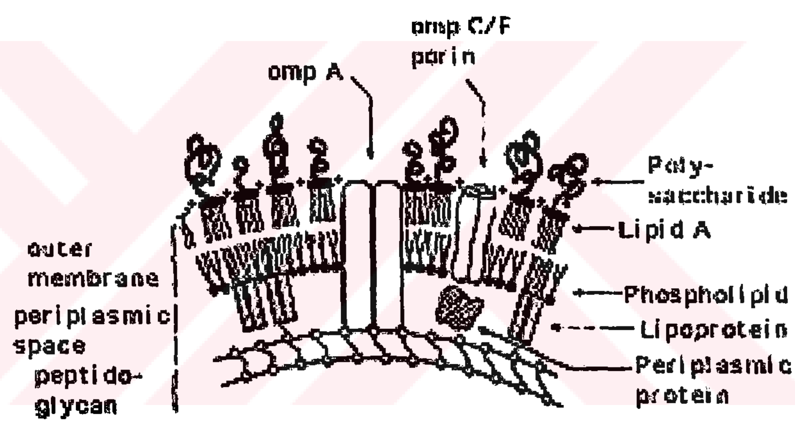


Figure 1.9. Structure of the cell surface of a Gram-negative bacterium (from Todar, 1997)

Gram-negative bacteria probably release minute amounts of endotoxin while growing. For example, it is known, that small amounts of endotoxin may be released in a soluble form, especially by young cultures. However, for the most part, endotoxins remain associated with the cell wall until disintegration of the bacteria. *In* *vivo*, this results from autolysis of the bacteria, external lysis mediated by complement and lysozyme, and phagocytic digestion of bacterial cells.

Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite, degrade them. Endotoxins, although strongly antigenic, cannot be converted to toxoids. A comparison of the properties of bacterial endotoxins and classic exotoxins is shown in Table 1.3.

Table 1.3. Characteristics of bacterial endotoxins and classic exotoxins . (from Todar, 1997)

PROPERTY	ENDOTOXIN	EXOTOXIN
CHEMICAL NATURE	Lipopolysaccharide (mw = 10kDa)	Protein (mw = 50-1000kDa)
RELATIONSHIP TO CELL	Part of outer membrane	Extracellular, diffusible
DENATURED BY BOILING	No	Usually
ANTIGENIC	Yes	Yes
FORM TOXOID	No	Yes
POTENCY	Relatively low (>100ug)	Relatively high (1 ug)
SPECIFICITY	Low degree	High degree
ENZYMATIC ACTIVITY	No	Usually
PYROGENICITY	Yes	Occasionally

Lipopolysaccharides participate in a number of outer membrane functions that are essential for bacterial growth and survival, especially within the context of a host-parasite interaction. An intact outer membrane exerts several vital functions in Gram-negative bacteria:

1. It is a permeability barrier that is permeable only to low molecular weight, hydrophilic molecules. In the Enterobacteriaceae, the ompF and ompC porins exclude passage of all hydrophobic molecules and any hydrophilic molecules greater than a molecular weight of about 700 daltons. This prevents penetration of the bacteria by bile salts and other toxic molecules from the GI tract. It also retains periplasmic components.

2. It impedes destruction of the bacterial cells by serum components and phagocytic cells.

3. It plays an important role as a surface structure in the interaction of the pathogen with its host. For example, LPS may be involved in adherence (colonization), or resistance to phagocytosis, or antigenic shifts that determine the course and outcome of an infection.

Most of the work on the chemical structure of endotoxin has been done with species of *Salmonella* and *E. coli.* LPS can be extracted from whole cells by treatment with 45% phenol at 90°. Mild hydrolysis of LPS yields Lipid A plus polysaccharide.

Lipopolysaccharides are complex amphiphilic molecules with a MW of about 10kDa, that vary widely in chemical composition both between and among bacterial species The general architecture of LPS is shown in Figure 1.10. The general structure of *Salmonella* LPS is shown in Figure 1.11 and the complete structure of *Salmonella* lipid A is illustrated in Figure 1.12.

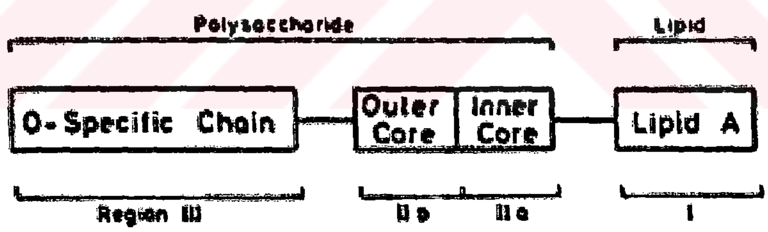


Figure 1.10. General architecture of Lipopolysaccharide (from Todar, 1997)

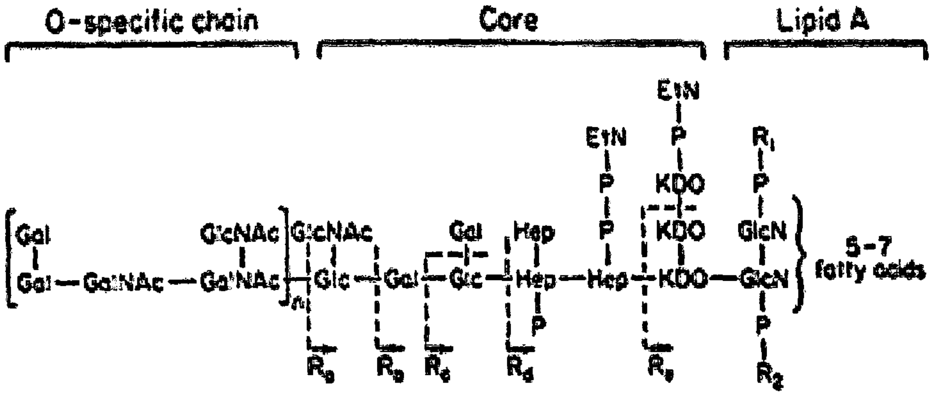


Figure 1.11. General structure of *Salmonella* LPS (from Todar, 1997)

Glc = glucose; GlcNac = N-acetyl- glucosamine; Gal = galactose; Hep = heptose; P = phosphate; Etn = ethanolamine; R1 and R2 = phoshoethanolamine or aminoarabinose. Ra to Re indicate incomplete forms of LPS. The Rd2 phenotype (not shown) would have only a single heptose unit. The Rc, Rd2, and Rd1 mutantslack the phosphate group attached to Hep.

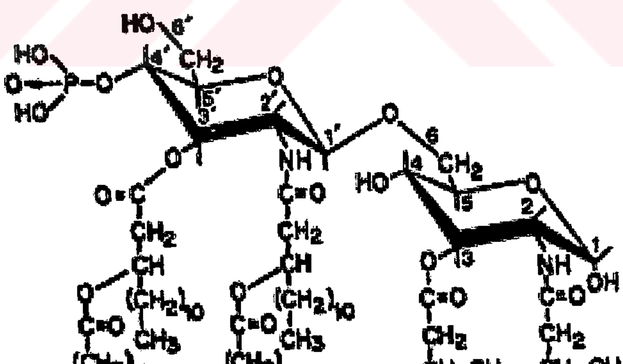


Figure 1.12. Complete structure of the Lipid A Moiety of LPS of *S. typhimurium*, *S. minnesota*, and *E. coli* (from Todar, 1997).

LPS consists of three components or regions:

Region I. Lipid A

Region II. Core (R) antigen

Region III. Somatic (O) antigen or O polysaccharide

1. Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram-negative bacteria. Among Enterobacteriaceae Lipid A is virtually constant.

2. Core (R) polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars.

For example: KDO - Hep - Hep – Glu - Gal - Glu - GluNAc –

Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctonoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin).

With minor variations, the core polysaccharide is common to all members of a bacterial genus (e.g. *Salmonella*), but it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores (Todar, 1997).

3. The O antigen or O side chain is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3 - 5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide. Great variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria. At least 20 different sugars are known to occur and many of these sugars are characteristically unique dideoxyhexoses, which occur in nature only in Gram-negative cell walls. Variations in sugar content of the O polysaccharide contribute to the wide variety of antigenic types of *Salmonella* and *E. coli* and presumably other strains of Gram-negative species. Particular sugars in the structure, especially the terminal ones, confer immunological specificity of the O antigen, in addition to "smoothness" (colony morphology) of the strain. Loss of the O specific region by mutation results in the strain becoming a "rough" (colony morphology) or R strain.

The structure of LPS in *Salmonella typhimurium* and *E. coli is* seen in Figure 1.10). The elucidation of the structure of LPS relied heavily on the availability of mutants each blocked at a particular step in LPS synthesis. The biosynthesis of LPS is strictly sequential. The core sugars are added sequentially to Lipid A by successive additions, and the O side chain is added last, one preassembled subunit at a time. The properties of mutants producing incomplete LPS molecules suggests the nature and biological functions performed by various parts of the LPS molecule(from Todar, 1997):

1. Loss of the O antigen results in loss of virulence suggesting that this portion is important during a host-parasite interaction. It is known that such "rough" mutants are more susceptible to phagocytosis and serum bactericidal reactions.

2. Loss of the more proximal parts of the core, as in "deep rough" mutants (i.e. in Rd1, Rd2, and Re mutants) makes the strains sensitive to a range of hydrophobic compounds, including antibiotics, detergents, bile salts and mutagens. This area contains a large number of charged groups and is thought to be important in maintaining the permeability properties of the outer membrane.

3. Mutants in the assembly of Lipid A cannot be isolated except as conditional lethal mutants and this region must therefore be essential for cell viability. The innermost region of LPS, consisting of Lipid A and three residues of KDO, appears to be essential for viability, presumably for assembling the outer membrane.

Both Lipid A (the toxic component of LPS) and the polysaccharide side chains (the nontoxic but immunogenic portion of LPS) act as determinants of virulence in Gram-negative bacteria. Virulence and the property of "smoothness" are regularly associated in many bacterial infections. The polysaccharide chain must also be important for virulence as shown by the fact that small changes in the sugar sequences in the side chains of LPS, result in major changes in virulence. How are the polysaccharide side chains involved in the expression of virulence? There are a number of possibilities:

a. Smooth antigens could allow organisms to adhere specifically to certain tissues, especially epithelial tissues.

b. Smooth antigens probably allow resistance to phagocytes, since rough mutants are more readily engulfed and destroyed by phagocytes.

c. The hydrophilic O polysaccharides could act as water-solubilizing carriers for toxic Lipid A. It is known that the exact structure of the polysaccharide can greatly influence water binding capacity at the cell surface.

d. The O antigens could provide protection from damaging reactions with antibody and complement. Rough strains of Gram-negative bacteria derived from virulent strains are generally non-virulent. Smooth strains have polysaccharide "whiskers" which bear O antigens projecting from the cell surface. The O antigens are the key targets for the action of host antibody and complement, but when the reaction takes place at the tips of the polysaccharide chains, a significant distance external to the general bacterial cell surface, complement fails to have its normal lytic effect. Such bacteria are virulent because of this resistance to immune forces of the host. If the projecting polysaccharide chains are shortened or removed, antibody reacts with antigens on the general bacterial surface, or very close to it, and complement can lyse the bacteria (Thus, "rough" colonial strains are non virulent.) (Todar, 1997).

1.14.2. Biological Activities of Endotoxins

Endotoxins are toxic to most mammals. Even though endotoxins are strong antigens, they seldom elicit immune responses which gives full protection to the animal against secondary challenge with the endotoxin. They cannot be toxoided. Regardless of the bacterial source, all endotoxins produce the same range of biological effects in the animal host.

Most of our knowledge of the biological activities of endotoxins derives not from the study of natural disease but by challenge of experimental animals.

The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as (i).fever (ii) changes in white blood cell counts (iii) disseminated intravascular coagulation (iv).tumor necrosis (v).hypotension (vi).shock (vii).lethality

Injection of large doses of endotoxin results in death in most mammals. The sequence of events follows a regular pattern: (1) latent period; (2) physiological distress (diarrhea, prostration, shock); (3) death. How soon death occurs varies on the dose of the endotoxin, route of administration, and species of animal. Animals vary in their susceptibility to endotoxin. Since Lipid A is embedded in the outer membrane of bacterial cells, it probably only exerts its toxic effects when released from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the Membrane Attack Complex (MAC), ingestion and killing by phagocytes, or killing with certain types of antibiotics. It is thought that LPS released into the bloodstream by lysing Gram-negative bacteria is first bound by certain plasma proteins identified as LPS-binding proteins. The LPS-binding protein complex interacts with CD14 receptors on monocytes and macrophages and other types of receptors on endothelial cells.

During infectious diseases caused by Gram-negative bacteria, endotoxins released from, or part of, multiplying cells have similar effects on animals and significantly contribute to the symptoms and pathology encountered. The range of inflammatory effects caused by LPS during Gram-negative bacteremia or septicemia are outlined below:

1. Complement activation: C3a and C5a cause histamine release (leading to vasodilation) and effect neutrophil chemotaxis and accumulation. The result is inflammation.

2. Initial activation of Hageman factor (blood-clotting Factor XII), which, in turn, can activate several humoral systems (Figure 1.6) resulting in:

(a) coagulation: a blood clotting cascade that leads to coagulation, thrombosis, acute disseminated intravascular coagulation, which depletes platelets and various clotting factors resulting in internal bleeding.

(b) activation of the complement alternative pathway (as above, which leads to inflammation)

(c) plasmin activation which leads to fibrinolysis and hemorrhaging.

(d) kinin activation releases bradykinins and other vasoactive peptides which causes hypotension. The net effect of LPS is to induce inflammation, intravascular coagulation, hemorrhage and shock.

3. LPS acts as a B cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM.

4. LPS activates macrophages to enhanced phagocytosis and cytotoxicity.
Macrophages are stimulated to produce and release lysosomal enzymes, Interleukin-I (IL-1) ("endogenous·pyrogen"), and tumor necrosis factor (TNF-alpha), as well as other cytokines and mediators.

These physiological activities of endotoxins are mediated mainly by the Lipid A component of LPS. The primary structure of Lipid A has been elucidated and Lipid A has been chemically synthesized. Its biological activity appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO4 groups, the acyl chains, and also the KDO-containing inner core. Thus Lipid A is a powerful biological response modifier that can stimulate the mammalian immune system.

1.15. Subdivision within Serovar: Genotypic Characterization

Genotypic subdivision of *Salmonella* is based on the characterization of the DNA of the organism either by analysis of the plasmid DNA content or by analysis of chromosomal DNA.

1.15.1. Plasmid Profile Typing

Plasmids isolated from *Salmonella* strains range in size from less than 1 megadalton (Mda) to 180 Mda. Plasmid typing for epidemiological purposes involves characterization of carried plasmids in terms of the numbers per strain and their molecular weights. The resultant 'plasmid profile' can be used for strain differentiation within serovar and within phage type.

The contribution of plasmids to the clinical and economic problems associated with salmonellosis is well recognized (Platt and Brown, 1986). Some of the plasmids appear to be indegenous to the genus (Terakado *et al.*, 1983; Nakamura *et al.*, 1985), whereas others, notably those that confer resistance to antimicrobial agents, have been largely acquired by conjugation from other members of the enterobacteria (Datta *et al.*, 1981; Platt *et al.*, 1984a). Since, in addition to phenotypic modification by plasmids, the possession of plasmids confers considerable genetic flexibility upon the host strains (Platt *et al.*, 1984b). It can be argued that a clearer understanding of plasmid ecology and their behaviour in the natural environment requires the establishment of the relative abundence (and distribution) of plasmids among defined collections of the enteric genera.

1.15.2. Plasmid Fingerprinting

A further degree of differentiation can be achieved by cleaving plasmid DNA, prepared as described above, with restriction endonucleases. These enzymes recognize specific DNA sequences, thereby cutting the plasmid into a number of fragments dependent on the number of enzyme recognition sites (Platt et al., 1987). The resultant plasmid 'fingerprint' increases the degree of differentiation provided by plasmid profile typing and is particularly useful in discriminating between plasmids of similar molecular weights. Plasmid fingerprinting also provides a measure of plasmid relatedness, and this can be useful in tracing the evalutionary origin of plasmids and of their host strains. A strategy of enzyme usage for the fingerprinting of enterobacterial plasmids has been proposed, with the aim of providing a fingerprint with a minimum of 12 restriction enzyme cleavage fragmentts (Platt *et al.*, 1986). This is based on the cleavage patterns obtained when plasmids in the strains under investigation are sequentially digested with the enzymes *Pst*1, *EcoR*1, *Hind*III, *Bsp*1286, *Ava*II and *Sma*I, used individually and in combination. The strategy has been successfully applied to fingerprinting plasmids in strains of *S. typhimurium* (Platt *et al.*, 1987).

Plasmid analysis can be further enhanced by using appropriately labelled single-stranded fragments of plasmid DNA as probes to demonstrate homology between plasmids of different molecular weights (Popoff *et al.*, 1984). In the preparation of single-stranded DNA probes, plasmid DNA carrying the required region is cleaved with a suitable restriction endonuclease and cloned into an appropriate host strain, using a plasmid or phage vector and standard transformation or transfection methods. The cloned fragments can then be sized, subcloned according to the degree of specificity required and labelled with an isotopic or non-isotopic compound by nick translation (Kelly *et al.*, 1970) or by random priming (Feinberg and Vogelstein, 1983, 1984). Denatured digested linear DNA fragments from the strains under investigation are transferred by blotting onto nitrocellulose paper or nylon membranes (Southern, 1975) and then hybridized with the selected probe or probes. Alternatively, digest fragments from the 'probe' plasmid may be separated by electrophoresis on agarose gels, excised form the gel, purified, labelled by random priming and used directly for hybridization analysis. Plasmid DNA probes have been used to demonstrate the widespread distribution of genes encoding resistance to variety of antibacterial drugs (Tenover, 1986) and the existence of a conserved 'virulence' region on apparently unrelated plasmids in several *Salmonella* serovars (Williamson *et al.*, 1988a; Woodward *et al.*, 1989).

1.15.3. Chromosomal Fingerprinting

Chromosomal DNA can be analysed by examination of restriction endonuclease cleavage fragment patterns in a similar way to plasmids (Roussel and Chabbert, 1978; Davis *et al.*, 1980). Linear DNA fragments obtained after digestion of chromosomal DNA with restriction endonucleases may then be separated by electrophoresis on agarose gels and visualized under *u.v.* illumination, after staining with an appropriate intercalating dye.

The fragment patterns obtained after digesting chromosomal DNA with restriction endonucleases are considerably more complex than those used for plasmid fingerprinting; pattern recognition can involve analysis of up to two hundred fragments of different sizes. Observation of pattern differences can be enhanced, however, by using pulsed field inversion electrophoresis (Schwartz and Cantor, 1984; Smith *et al.*, 1987). This method of electrophoretic analysis can provide increased resolution between linear fragments within a predefined size range.

Observations of heterogeneity in chromosomal restriction fragment patterns can also be enhanced by using single-stranded chromosomal DNA probes (Tompkins *et al.*, 1986). These can be prepared from chromosomal DNA fragments from a suitable strain and labelled with an isotopic or non-isotopic compound as described above. Digested chromosomal DNA from the strains under investigation may then be denatured, transferred to nitrocellulose paper or nylon filters and hybridized with labelled probe DNA, thereby demonstrating the presence or absence of homologous sequences.

Radiolabelled ribosomal nucleic acids (rRNA) have also been used to enhance analysis of endonuclease-cleaved chromosomal DNA (Grimont and Grimont, 1986). By this method, ribosomal 16 and 23S rRNA which has been extracted from *Escherichia coli* and isotopically labelled, is used to probe preparations of restriction endonuclease-cleaved chromosomal DNA. Variation in copy number and size of fragments carrying rRNA genes (rDNA) can then be detected, and this may provide another method of strain discrimination.

1.15.4. Virulence Properties

The 38 Mda plasmid carried by the majority of strains of *S. enteritidis* (Helmuth *et al.*, 1985) has been shown to encode mechanisms which are essential for establishing experimental infections in mice (Helmuth *et al.*, 1985; Chart *et al.*, 1989). Because of its widespread distribution in strains of *S. enteritidis* from widely separated geographical areas, this 38 Mda plasmid has been designated *S. enteritidis* 'serotype-specific' plasmid (SSP) (Helmuth *et al.*, 1985). Spontaneous loss of the SSP from strains of *S. enteritidis* PT 4 results in a million-fold increase in the Lethal Dose-50 (LD^{50}) (Chart *et al.*, 1989). Although the mechanisms involved in mouse virulence have not been determined, much of speculation has focused on the ability of SSP-carrying strains to survive intracellularly in mouse phagocytes. The *S. enteritidis* SSP has classically been termed the 'virulence' plasmid (Helmuth *et al.*, 1985). However, recent studies have demonstrated that although this plasmid is indisputably involved in the virulence of *S enteritidis* for mice, it does not appear to be involved in the pathogenicity of the organism for poultry (Hinton *et al.*, 1990). Furthermore, strains of *S.enteritidis* carrying the SSP are only pathogenic for strains of inbred mouse, for example BALB/c, which do not evoke an immune response to bacterial LPS (Chart and Rowe, 1991). It is also noteworthy that strains of *S. enteritidis* PT 4 which do not carry the SSP have been isolated from humans with gastroenterititis, and an SSP-strain of *S. enteritidis* PT 4 has been identified as the causative agent in a hospital outbreak of food-poisoning in Wales in 1990.

DNA-DNA hybridization studies performed in several laboratories have demonstrated the existance of considerable homology in the SSPs of *S. enteritidis*, *S.*

typhimurium, S. dublin and *S. cholerae-suis* (Popoff *et al.*, 1984; Williamson *et al.*, 1988; Poppe *et al.*, 1989) and the regions responsible for virulence in the *S. typhimurium, S. dublin* and *S. cholerae-suis* SSPs have been sequenced (Norel *et al.*, 1989; Pullinger *et al.*, 1989; Matsui *et al.*, 1990). In these plasmids the genes responsible for virulence for BALB/c mice, now designated the *Salmonella* plasmid virulence (*Spv*) genes, are encoded on a 3.5 kb *Hind* III fragment which has been shown to be carried on 38 Mda plasmids found in 18 of the *S. enteritidis* phage type strains, on 59 Mda plasmids identified in 6 of the phage type strains and on a 45 Mda plasmid found in the type strain of *S. enteritidis* PT 19 (Brown *et al.*, 1993).

The ability of strains of *S. enteritidis* to express LPS has also been recognized as a virulence property. Strains of *S. enteritidis* which express long-chain LPS and carry the SSP have an LD^{50} for BALB/c mice of between 10 and 20 bacteria (Chart *et al.*, 1989); in contrast, strains of *S.enteritidis* which carry this plasmid but are unable to produce LPS are avirulent for BALB/c mice (Chart *et al.*, 1989).

Strains of *S. enteritidis* have been shown to express an enterobactin-mediated iron uptake system (Chart *et al.*, 1989; Chart and Rowe, 1993). The possession of a high affinity iron sequestring system makes strains able to compete with chelators, such as egg ovotransferrin, for ferric ions (Chart and Rowe, 1993).

1.15.5. IS*200* Typing

IS*200* is a *Salmonella*-specific insertion element first described by Lam and Roth (1983) and distributed on conserved loci on the chromosome of many

Salmonella serotypes (Gibert *et al*., 1990). Certain characteristics of IS*200* favour its use as a probe for strain discrimination and using IS*200*, three 'clonal lineages', designated SEC1I, SEC1II and SEC1III have been identified within the chromosomes of 27 of the *S. enteritidis* phage type strains (Stanley *et al*., 1991). The type strain of *S. enteritidis* PT 4 belonged to SEC1I and, following chromosomal digestion with the restriction endonuclease *Pst* I, was characterized by two IS*200* loci of 4.5 and 5.2 kb (Stanley *et al*., 1991). However IS*200* typing was unable to differentiate 29 strains of *S. enteritidis* PT 4 isolated from humans, poultry and poultry products in five countries over a 20-year period (Stanley *et al*., 1992a; Stanley *et al*., 1992b), although some of these strains could be subdivided on the basis of plasmid profiles (Stanley *et al*., 1992b).

1.16. Interrelationship between Strains of *Salmonella enteritidis*

The possible existence of interrelated strains of *S. enteritidis* belonging to different phage types was indicated when smooth (LPS^+) strains of *S. enteritidis* belonging to PT 4 were shown to spontaneously mutate to rough (LPS^-) strains of *S. enteritidis* belonging to PT 7 (Chart *et al*., 1989). This irreversible mutation occurred at a rate of approximately 1 in 10, and converted strains of *S. enteritidis* PT 4, virulent for BALB/c mice to avirulent strains of *S. enteritidis* belonging to PT 7 (Chart *et al*., 1989).

Phage type conversion was also demonstrated by experiments involving plasmid transfer. Strains of *S. enteritidis* belonging to PT 4 were shown to convert to strains of *S. enteritidis* belonging to PT 24 following the acquisition of an *Inc* N drug resistance plasmid (Frost *et al.*, 1989). More recent studies (Threlfall and Chart, 1993) have shown that transferring pDEP 44, a 34 Mda *Inc* N plasmid coding for resistance to ampicillin and streptomycin, to strains of *S enteritidis* belonging to PTs 7, 7a, 8, 13, 13a, 24 and 30 demonstrated that the phage type designation of certain other phage types of *S. enteritidis* could also be altered by the acquisition of *Inc* N plasmids (Figure 1.13).

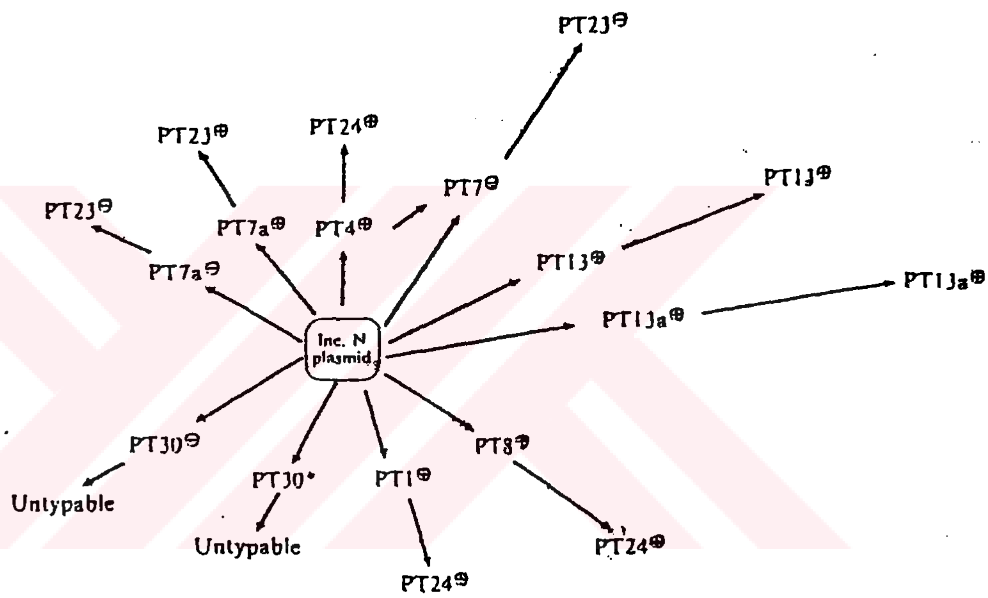


Figure 1.13. Diagramatic representation showing the interactions between strains of *S. enteritidis*, and in particular the role of *Inc* N plasmids in changing phage type (PT). Strains of *S. enteritidis* belonging to PT 4 were shown to convert to strains of *S. enteritidis* belonging to PT 24 following the acquisition of pDEP 44, an Inc N drug resistance plasmid coding for resistance to A and S. Also, tranferring pDEP 44 to strains of *S. enteritidis* belonging to PTs 1, 7, 7a, 8, 13, 13a, 24 and 30 demonstrated that the phage

type designation of certain other phage types of *S. enteritidis* could also be altered by the acquisition of *Inc* N plasmids. Strains of *S. enteritidis* with long-chain LPS (+), with no LPS (-) or trace amounts of LPS(*) (from Threlfall and Chart, 1993).

The ecquisition of pDEP 44 by strains of *S. enteritidis* PT 4 caused these strains to convert to smooth strains of *S. enteritidis* PT 24. Acquisition of pDEP 44 also resulted in the conversion of strains of *S. enteritidis* of PT 1 to PT 24 (unpublished), *S. enteritidis* of PTs 7 and 7a to *S. enteritidis* PT 23, *S. enteritidis* PT 8 to PT 24 and *S. enteritidis* PT 30 to a strain of *S. enteritidis* which did not react with the typing phages and was therefore designated untypable. The reactions of the strains of *S. enteritidis* PTs 13 and 13a with the phages which lyse strains of these phage types were lowered but the reduction in lysis was not sufficient to warrant alteration in phage type designation. Acqusition of pDEP 44 did not result in the displacement of the *S. enteritidis* SSP nor of any low MW plasmids found in some of the wild-type isolates of the respective phage types (Threlfall *et al.*, 1993).

Both wild type and exconjugant strains of *S.enteritidis* PTs 1, 4, 8, 13 and 13a into which pDEP 44 had been introduced expressed LPS. In contrast neither the wild-type strain of *S. enteritidis* PT 7, nor the exconjugant strain of *S. enteritidis* PT 23 derived from *S. enteritidis* PT 7a, expressed LPS. Strain of *S. enteritidis* PT 7a are comprised of strains which either express long-chain LPS or do not express LPS (Threlfall *et al.*, 1993). Rough and smooth strains of *S. enteritidis* PT 7a have been isolated from humans and ducks but in contrast, only rough strains of this phage type have been obtained from chickens. Transfer of pDEP 44 to rough strains of *S. enteritidis* PT 7a , result in the formation of smooth strains of *S. enteritidis* which also phage type as PT 23 and express LPS (Threlfall *et al.*, 1993). *S. enteritidis* PT 30 is comprised of strains which either make trace amounts of long chain LPS or do not express LPS. Regardless of whether strains express LPS or not, acquisition of pDEP 44 results in the formation of strains of *S. enteritidis* which become untypable by phages. The role of pDEP 44 in changing binding sites for typing phages remains to be elucidated. The importance of long-chain LPS in determining phage type in strains of *S. enteritidis* has been reported (Chart *et al.*, 1989). However carriage of pDEP 44 may alter expression of LPS, altough profiles so far obtained using SDS-PAGE do not suggest this.

To date, phage typing has been of paramount importance in studying the epidemiology of this serotype and in particular, has demonstrated the involvement of both poultry meat and whole shell eggs in the transmission of *S. enteritidis* PT 4 to humans. The findings discussed above describe various aspects of serotype, particularly in relation to the involvement of both LPS and plasmids in its virulence and phage type identity (Figure 1.15). These findings have led to an increased understanding of the biology of this serotype, which is of major importance in human food-poisoning in the world.

The subdivision of *S. typhimurium* by plasmid profile typing and phage typing has been compared and results of two studies indicated that plasmid profile typing was at least as specific as phage typing (lysotyping) in confirming the identity of epidemiologically related strains, and considerably more discriminatory than resistance typing and biotyping in this context (Brunner *et al.*, 1983; Holmberg *et al.*, 1984). In contrast, examination of the plasmid content of a limited number of unrelated strains of *S. typhimurium* belonging to 16 phage types demonstrated that a high proportion of strains harboured only a single 60 MDa plasmid, characterized by a distinctive restriction endonuclease fingerprint (Brown *et al.*, 1986).

Analysis of the plasmid content of strains belonging to different serovars has revealed that several other serovars carry plasmids which can be regarded as 'serovar-specific'. Serovars of epidemiological importance in humans and food animals which harbour such plasmids include *S. typhimurium, S. dublin, S. enteritidis, S. cholerae-suis* (Helmuth *et al.*, 1985) and *S. pullorum* (Barrow and Lovell, 1988). These serovar-specific plasmids have molecular weight ranging from about 30 Mda in *S. cholerae-suis*, to 62 Mda in *S. typhimurium* (Helmuth *et al.*, 1985).

The results of animal challenge experiments have indicated that these serovar-specific plasmids are involved in the virulence of their bacterial hosts for mice (Helmuth *et al.*, 1985; Baird *et al.*, 1985; Chart *et al.*, 1989a). When cleaved with restriction endonucleases, virulence plasmids from the same serovar were found to be homogeneous, whereas plasmids from different serovars had markedly different cleavage patterns. DNA-DNA hybridization, however, demonstrated that homologous sequences were distributed throughout these serovar-specific plasmids (Popoff *et al.*, 1984). Furthermore, an 8 kb fragment of *S. dublin* serovar-specific plasmid has been found to have the ability to restore virulence to plasmid-cured strains of *S. typhimurium* and *S. dublin* (Williamson *et al.*, 1988b), and a 4 kb fragment from within this region has been shown to have homology with regions of the serovar-specific plasmids of *S. typhimurium* and *S. enteritidis* (Woodward *et al.*, 1989). These experimental findings indicate that a highly conserved region apparently involved in certain aspects of *Salmonella* virulence is distributed in otherwise unrelated plasmids belonging to several serovars. This could allow for the development of a specific DNA probe for *Salmonella* virulence, which might be a useful tool in some epidemiological investigations.

Subdivision of *S. enteritidis* by plasmid profile typing has also been compared to phage typing. The results showed that plasmid profile typing was no as sensitive as phage typing for the primary differentiation of this serovar (Threlfall *et al.*, 1989). Nevertheless, considerable discrimination within some phage types was achieved, including differentiation within the two most common phage types in England and Wales, phage types 4 and 8.

Strain differentiation based on the genotypic characterization of plasmid DNA can be used only in serovars or phage types which harbour several plasmids of different molecular weights. It is of limited value in serovars such as *S. infantis* or *S. panama* (Helmuth *et al.*, 1985) and *S. ealing* (Threlfall, unpublished observations), in which the majority of strains are plasmid-free. Similarly, the method cannot satisfactorily be used to subdivide serovars such as *S. cholerae-suis*, *S. enteritidis* or *S. dublin*, where the majority of strains carry only the serovar-specific plasmid.

One of the main advantages of chromosomal (genomic) fingerprinting is that it can be applied to the differentiation of strains which do not carry plasmids. *S. typhi* is a serovar in which the majority of strains are plasmid-free (Murray *et al.*, 1985). Chromosomal fingerprinting, using four restriction endonucleases, has been used to compare strains of *S. typhi* isolated in Chile, Peru and the United States. No differences were detected when genomic DNA preparations were cleaved with three of the four enzymes, but small differences observed amongst strains from Peru and Chile after digestion with one enzyme, *Pst* 1 (Maher *et al.*, 1986).

^{32}P-labelled fragments of total cell DNA from a strain of *S. enteritidis* have been used as probes to recognize chromosomal restriction fragment length polymorphism (RFLPs) in strains of *S. typhimurium*, *S. dublin* and *S. enteritidis* from outbreaks in the United States. Using this method, strains of *S. typhimurium* were grouped into six major types which corresponded with epidemiological groupings, and six fingerprint patterns were observed in isolations of *S. dublin*. In contrast, the majority of strains of *S. enteritidis* had a homogenous probe fingerprint, which indicates that there has been little evalutionary divergence in this serovar (Tompkins *et al.*, 1986).

As yet, the use of ribosomal nucleic acid probes to identify patterns of chromosomal rRNA genes within a serovar has not been applied to outbreak investigations. The method does enable discrimination at the subspecies level (Grimont and Grimont, 1986), however, and may be of use for the subdivision of some plasmid-free serovars.

Since the 1930s, traditional methods of strain identification based on serotyping and phage typing have been the foundation of *Salmonella* epidemiology. Although the incidence of diseases such as typhoid and paratyphoid has decreased in recent years, food-poisoning caused by non-typhoidal *Salmonella* strains has now reached epidemic proportions in many countries, despite improvements in sanitation and hygiene. Precise strain identification is an essential prerequisite for epidemiological investigations aimed at combating the spread of these strains and eradicating the sources of infection.

Modern methods of genotypic typing, particularly those based on physical characterization of the plasmid content of the organism have already proved invaluable for the identification and differentiation of strains in many outbreaks. These plasmid typing methods are now increasingly used with serotyping and phage typing for many epidemiological investigations. Other methods of genotypic typing, particularly those based on recognition of small differences in chromosome structure, are not yet practical for the examination of large number of strains. Nevertheless, improvements in small scale methods for chromosomal DNA extraction coupled with the increasing use of non-isotopic labels for identification of restriction fragment length polymorphisms may provide a new dimension to *Salmonella* epidemiology.

In Turkey, the isolation of *S. enteritidis* from cases of gastroenteritis has increased noticeably in the past decade (Anğ *et al.*, 1993; Erdem *et al.*, 1994). Yıldırmak and his colleagues (1998) showed the antibiotic resistance and plasmid profiles of clinical isolates of *S. typhimurium* from the country. The antibiotic resistance profiles of *S. typhimurium* isolates were also studied by Arman in 1993. However, because of the lack of a national reference centre, no statistical data are available and phage typing has not yet been attempted.

The present study was conducted to characterize at a molecular level 82 *Salmonella enteritidis* strains isolated from eggs, chickens and humans. At present, there is a paucity of information to explain increased prevalence of *S. enteritidis* in people and animals or to propose that the increased occurrence of *S. enteritidis* infections may be due to a change in characteristics of the pathogen. The aims of this thesis were:

(i) to characterize *S. enteritidis* strains with respect to plasmid profile (PP), resistance to antimicrobial agents, phage types (PT), outer membrane protein (OMP) profiles and lipopolysaccharide (LPS) profiles,

(ii) to determine how these characteristics relate to one another,

(iii) to examine if these traits might have diagnostic value or might explain pathogenic or epidemiological findings, and

(iv) to compare our findings to those obtained from other *Salmonella* serovars documented in the literature.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains

Eighty-two isolates of *S. enteritidis* were used in this study. Of these, twenty-one chicken isolates were obtained from three sources; Numune Hospital, Lalahan

Table 2.1. *S. enteritidis* chicken isolates used in this study

Strain Designation	Area of Isolation	Supplier
A1	Ankara	Numune Hospital, Dept. Bacteriol.
A2	Ankara	Numune Hospital, Dept. Bacteriol.
A3	Ankara	Lalahan Animal Health Institute
A4	Ankara	Numune Hospital, Dept. Bacteriol.
A5	Ankara	Numune Hospital, Dept. Bacteriol.
A7	Ankara	Numune Hospital, Dept. Bacteriol.
A8	Ankara	Numune Hospital, Dept. Bacteriol.
A9	Ankara	Numune Hospital, Dept. Bacteriol.
A10	Ankara	Numune Hospital, Dept. Bacteriol.
A11	Ankara	Numune Hospital, Dept. Bacteriol.
A12	Ankara	Numune Hospital, Dept. Bacteriol.
A13	Ankara	Lalahan Animal Health Institute
A14	Ankara	Numune Hospital, Dept. Bacteriol.
A15	Ankara	Numune Hospital, Dept. Bacteriol.
A16	Ankara	Numune Hospital, Dept. Bacteriol.
A17	Ankara	Numune Hospital, Dept. Bacteriol.
A19	Ankara	Numune Hospital, Dept. Bacteriol.
A22	Ankara	Lalahan Animal Health Institute
A23	Ankara	Numune Hospital, Dept. Bacteriol.
A25	Bursa	Uludağ Univ. Dept. of Microbiol.
A26	Bursa	Uludağ Univ. Dept. of Microbiol.

Animal Health Institute-Ankara and Uludağ University-Bursa, and designated A1, A2, A3, A4, A5, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A17, A19, A22, A23, A25 and A26, respectively (Table 2.1).

Fifty-one human isolates were obtained from Numune Hospital, Department of Bacteriology; Ankara University Department of Biology, Culture Collection Centre; Numune Hospital Department of Clinical Microbiology-Ankara and Uludağ University, Faculty of Medicine. The isolates were designated H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, H18, H19, H20, H21, H22, H24, H25, H26, H27, H30, H34, H35, H36, H37, H39, H40, H41, H42, H43, H44, H45, H46, H47, H48, H50, H52, H53, H55, H56, H57, H58, H59, H62 and H63, respectively (Table 2.2).

Ten egg isolates were provided by Ankara-Kazan Köytur and Uludağ University, Faculty of Medicine and were designated E1, E3, E4, E7, E8, E9, E10, E11, E12 and E13, respectively (Table 2.3).

Table 2.2. *S. enteritidis* human isolates used in this study

Strain Designation	Area of Isolation	Supplier
H1	Ankara	Numune Hospital, Dept. Bacterio.
H2	Ankara	Numune Hospital, Dept. Bacterio.
H3	Ankara	Numune Hospital, Dept. Bacterio.
H4	Ankara	Numune Hospital, Dept. Bacterio.
H5	Ankara	Numune Hospital, Dept. Bacterio.
H6	Ankara	Numune Hospital, Dept. Bacterio.
H7	Ankara	Numune Hospital, Dept. Bacterio.
H8	Ankara	Numune Hospital, Dept. Bacterio.
H9	Ankara	Numune Hospital, Dept. Bacterio.
H10	Ankara	Numune Hospital, Dept. Bacterio.
H11	Ankara	Numune Hospital, Dept. Bacterio.
H12	Ankara	Numune Hospital, Dept. Bacterio.
H13	Ankara	Ankara Univ. Dept. Biology
H14	Ankara	Ankara Univ. Dept. Biology
H15	Ankara	Ankara Univ. Dept. Biology
H16	Ankara	Ankara Univ. Dept. Biology
H17	Ankara	Ankara Univ. Dept. Biology
H18	Ankara	Ankara Univ. Dept. Biology
H19	Ankara	Ankara Univ. Dept. Biology
H20	Ankara	Numune Hospital, Dept. Microbiol.
H21	Ankara	Numune Hospital, Dept. Microbiol.
H22	Ankara	Numune Hospital, Dept. Microbiol.
H24	Ankara	Numune Hospital, Dept. Microbiol.
H25	Ankara	Numune Hospital, Dept. Microbiol.
H26	Ankara	Hıfzı Sıhha
H27	Bursa	Uludağ Univ. Fac. Medicine
H30	Bursa	Uludağ Univ. Fac. Medicine
H34	Bursa	Uludağ Univ. Fac. Medicine
H35	Bursa	Uludağ Univ. Fac. Medicine
H36	Bursa	Uludağ Univ. Fac. Medicine
H37	Bursa	Uludağ Univ. Fac. Medicine
H39	Bursa	Uludağ Univ. Fac. Medicine
H40	Bursa	Uludağ Univ. Fac. Medicine
H41	Bursa	Uludağ Univ. Fac. Medicine
H42	Bursa	Uludağ Univ. Fac. Medicine
H43	Bursa	Uludağ Univ. Fac. Medicine
H44	Bursa	Uludağ Univ. Fac. Medicine
H45	Bursa	Uludağ Univ. Fac. Medicine
H46	Bursa	Uludağ Univ. Fac. Medicine
H47	Bursa	Uludağ Univ. Fac. Medicine
H48	Bursa	Uludağ Univ. Fac. Medicine
H50	Bursa	Uludağ Univ. Fac. Medicine
H52	Bursa	Uludağ Univ. Fac. Medicine
H53	Bursa	Uludağ Univ. Fac. Medicine
H55	Bursa	Uludağ Univ. Fac. Medicine
H56	Bursa	Uludağ Univ. Fac. Medicine
H57	Bursa	Uludağ Univ. Fac. Medicine
H58	Bursa	Uludağ Univ. Fac. Medicine
H59	Bursa	Uludağ Univ. Fac. Medicine
H62	Bursa	Uludağ Univ. Fac. Medicine
H63	Bursa	Uludağ Univ. Fac. Medicine

Table 2.3. *S. enteritidis* egg isolates used in this study

Strain Designation	Area of Isolation	Supplier
E1	Ankara	Köytur
E3	Ankara	Köytur
E4	Ankara	Köytur
E7	Ankara	Köytur
E8	Bursa	Uludağ Univ. Dept. Microbiol.
E9	Bursa	Uludağ Univ. Dept. Microbiol.
E10	Bursa	Uludağ Univ. Dept. Microbiol.
E11	Bursa	Uludağ Univ. Dept. Microbiol.
E12	Bursa	Uludağ Univ. Dept. Microbiol.
E13	Bursa	Uludağ Univ. Dept. Microbiol.

Twenty-two enteric species other than S. enteritidis that were also used in this study are shown in Table 2.4 below.

Table 2.4. Other enteric isolates used in the study

Strain Designation	Name of the Species	Source	Area of Isolation	Suplier
E1	*S. kedougou*	Egg	Ankara	Köytur
E5	*Salmonella* sp.	Egg	Ankara	Köytur
E6	*Enterobacter* sp.	Egg	Ankara	Köytur
A6	*S. typhimurium*	Chicken	Ankara	Numune Hospital, Dept. Bacteriol.
A18	*S. ouakam*	Chicken	Ankara	Lalahan Animal Health Institute
A20	*S. typhimurium*	Chicken	Ankara	Numune Hospital, Dept. Bacteriol.
A21	*S. ouakam*	Chicken	Ankara	Lalahan Animal Health Institute
A24	*S. ouakam*	Chicken	Ankara	Numune Hospital, Dept. Bacteriol.
H23	*S. baildon*	Human	Ankara	Ankara Univ. Dept. Biology
H28	*S. typhimurium*	Human	Bursa	Uludağ Univ. Fac. Medicine
H29	*S. typhimurium*	Human	Bursa	Uludağ Univ. Fac. Medicine
H31	*S. typhimurium*	Human	Bursa	Uludağ Univ. Fac. Medicine
H32	*S. havana*	Human	Bursa	Uludağ Univ. Fac. Medicine
H33	*S. typhimurium*	Human	Bursa	Uludağ Univ. Fac. Medicine
H38	*S. agona*	Human	Bursa	Uludağ Univ. Fac. Medicine
H49	*S. typhimurium*	Human	Bursa	Uludağ Univ. Fac. Medicine
H51	*Citrobacter freundii*	Human	Bursa	Uludağ Univ. Fac. Medicine
H54	*Enterobacter*	Human	Bursa	Uludağ Univ. Fac. Medicine
H60	*Proteus*	Human	Bursa	Uludağ Univ. Fac. Medicine
H61	*Citrobacter* sp.	Human	Bursa	Uludağ Univ. Fac. Medicine
V517	*E. coli*	-	-	M.E.T.U.
K12	*E. coli*	-	-	M.E.T.U.

Serological tests and phage typing for all the isolates were confirmed by the Central Public Health Laboratory (CPHL) Laboratory of Enteric Pathogens, Colindale, United Kingdom.

2.2. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are listed in Appendix C.

2.3. Culture Media

Composition and preparation of culture media are given in Appendix A.

2.4. Maintenance of Bacterial Strains

The strains were maintained as frozen stocks in LB broth (Luria-Bertani) supplemented with 12% glycerol at -70°C.

2.5. Buffers and Solutions

Composition of the buffers and other solutions is presented in Appendix B.

2.6. Resistance to Antimicrobial Agents

The disc diffusion method (NCCLS, 1997) that has been accepted by the Food and Drug Administration (FDA) and the National Committee for Clinical Laboratory Standards (NCCLS) was used in this study.

Dics containing wide variety of antimicrobial agents were applied to the surface of agar plates inoculated with pure cultures of clinical isolates of *S. enteritidis*. Following incubation, the plates were examined and the zones of inhibition surrounding the discs were measured and compared with prestablished zone size ranges for individual antimicrobial agents in order to determine antibiotic resistance patterns of *S. enteritidis* isolates. The following antibiotics were used to test all strains for resistance: amikacin (30µg), amoxicillin/clavunalic acid (20/10µg), ampicillin (10µg), aztreonam (30µg), carbenicillin (100µg), cefaclor (30µg), cephalothin (30µg) , cefoperazone (75µg), ceftazidime (30µg), ceftriaxone (30µg), cefuroxime (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), gentamicin (120µg), imipenem (10µg), oflaxacin (5µg), piperacillin (100µg), cefixime (5µg), sulperazone (75µg), ampicillin/sulbactam (10/10µg), tetracyclin (30µg), tobramycin (10µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), cefodizime (30µg) and meropehem (10µg).

2.7. Reference Plasmid Marker

A supercoiled DNA ladder (Sigma D5292) and *E. coli* V517 were used as the markers. The reference ladder possessed 11 plasmids with the following sizes in kilobase (kb); 2.067, 2.972, 3.990, 5.012, 6.030, 7.048, 8.066, 10.102, 14.174, and 16.210, respectively. *E. coli* V517 possessed 9 distinct plasmids with the following sizes in kb; 2.1, 2.7, 3.1, 3.9, 5.1, 5.6, 7.2, and 54.1, respectively.

A standard curve was constructed for each gel using the molecular weights of standard markers and the distance they migrated in the gel. The standard curve was used to calculate the molecular weight of each plasmid in the isolates of *S. enteritidis* (Fig. 2.1).

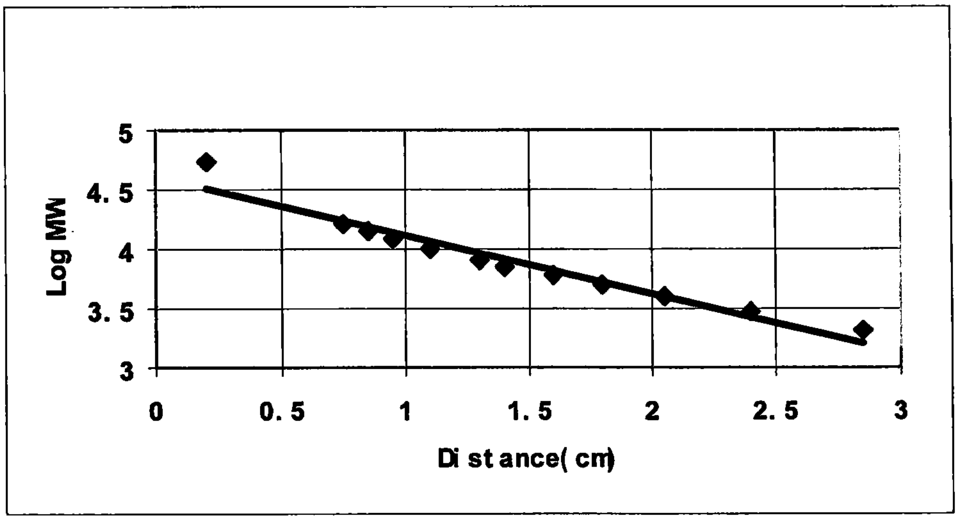


Figure 2.1. Standard curve for molecular weight estimation for the plasmids

2.8. Plasmid Isolation

Plasmid DNA was isolated by using a modification of the method of Kado and Lui (1981), using 1.5 mL volumes of strains grown at 37°C with gentle aeration for 18h. After centrifugation and resuspension in 20 μL of 50 mM TrisCl/1mM EDTA, pH 8.0, cells were lysed by incubation for 30 min. at 55°C with 0.05 M Tris/3 % SDS at pH 12.5. Chromosomal DNA, protein/SDS complexes, RNA and cell wall debris were removed by extraction with TE-saturated phenol/chloroform and 50 μL of the resultant plasmid DNA solution was analysed by electrophoresis.

2.9. Chromosomal DNA Isolation

Chromosomal DNA of bacterial strains was isolated by using the procedure of Cutting and Horn, (1990). 15 mL cultures was centrifuged at 5000 rpm for 10 min. Pellet was resuspended in 5.7 mL of TE buffer by repeated pipetting. Then 300 μL of

10% SDS, 30 μL of 20 mg/mL proteinase K and Rnase were added, mixed and incubated for 1 h at 37°C. 1000 μL of 5M NaCl was added and mixed throughly, 800 μL of CTAB/NaCl solution was added and the next mixture was incubated for 10 min at 65°C. It was then extracted with chloroform/isoamyl alcohol and microfuged for 5 min at 13000 rpm. The supernatant was tranferred to a new tube and reextracted with phenol/chloroform/isoamyl alcohol and microfuged for 5 min at 13000 rpm. 0.6 volume isopropanol was added to the upper phase, mixed and microfuged for 10 min. 5 mL of 70% ethanol was next added to the pellet and microfuged for 10 min. Pellet was dried and dissolved in 200 μL of TE buffer and stored at -20°C.

2.10. Agarose Gel Electrophoresis

The DNA preparations were electrophoresed in 0.7% agarose gels with Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA (pH 7.6) at 35 mA (110V.) for 2.5 h at room temperature. The gels were stained with ethidium bromide for 20 min. and destained with distilled water for 30 min. Photographs af the DNA bands were taken under UV light using a MP-4 polaroid camera.

2.11. Preparation of LPS

Preparation of LPS was performed by proteinase K digestion (Hitchcock and Brown, 1983). Briefly, bacterial growth was scraped from agar plates with 1 mL of phosphate-buffered saline (2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 145 mM NaCl) per plate and incubated in a tube at 60°C for 20 min. Following incubation the samples were centrifuged, and 50 μL of the supernatant was incubated with 50 μL of sample buffer (1 M Tris, 0.8 mM EDTA, 1% SDS, 5% β-mercaptoethanol, 22% glycerol, 0.01% bromophenol blue) at 100°C for 10 min. The preparation was mixed with proteinase K at a final concentration of 0.4 mg/mL and incubated at 60°C for 60 min. Following incubation, preparations were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.12. SDS-PAGE for LPS

LPS preparations were subjected to SDS-PAGE with the Laemmli buffer system (Laemmli, 1970) using a 16.5% resolving gel and a 4% stacking gel. Gels were run in a Mini-Protean II system (Bio-Rad) at 50 V for 20 min and 200 V for 1 h, after which gels were removed and stained with silver (Morrissey, 1981).

2.13. Silver Staining

The sensitivity of silver stain is sufficiently high so as to require careful cleaning of glass electrophoresis plates and glass staining dishes. The equipment and all avessories were therefore washed with detergent and warm water and rinsed throughly. In all steps, gels were agitated on an orbital shaker.

The procedure used for Ag-LPS staining included the following steps: (i) overnight fixation in 200 mL of 25% (vol/vol) isopropanol in 7% (vol/vol) acetic acid; (ii) 5-min oxidation in 150 mL of distilled water with 1.05 g of periodic acid and 4 mL of 25% (vol/vol) isopropanol in 7% (vol/vol) acetic acid (solution made up just before use); (iii) eight times 30min washes, each with 200 mL of distilled water; (iv) 10-min silver staining in a solution consisting of 0.1 N NaOH (28 mL) concentrated (29.4%) ammonium hydroxide (1 mL), 20% (wt/vol) silver nitrate (5 mL), and distilled water (115 mL) (make up solution just before use and stir constantly while making); (v) four times 10min washes, each with 200 mL of distilled water; (vi) 10 to 20 min of developing in 250 mL of developer solution (citric acid [50 mg] , 37% formaldehyde [0.5 mL], distilled water [amount sufficient to make 1 liter of solution]; made up just before use) at a optimal temperature of 25°C (if solution cools, staining of protein as well as LPS will occur); (vii) 1 h in a stop bath (200 mL of distilled water plus 10 mL of 7% (vol/vol) acetic acid); (viii) final wash with 200 mL of distilled water and then storage (gel may be stored in a zip-lock plastic bag with a small amount of water to prevent desiccation)

Use of concentrated (29.4%) ammonium hydroxide is essential for the staining of LPS. To maintain the quality of this reagent, we place small amounts of ammonium hydroxide are placed from a freshly opened bottle into small (25 to 50 mL) bottles with caps that can be tightly sealed. Loss of strength of the reagent results in persistence of the brown precipitate in the staining solution.

On the other hand, the Ag-LPS and protein staining procedure is identical to the Ag-LPS staining procedure, except for the followings: in step ii (5min oxidation), 10 mL of 40% (vol/vol) ethanol in 5% (vol/vol) acetic acid was added to the water-periodic acid solution. In step iv (silver staining), 3 mL of ammonium hydroxide was used (concentrated ammonium hydroxide was diluted with distilled water to 75% [vol/vol] of the original concentration [final concentration, 22%]).

2.14. Isolation of Outer Membrane Proteins (OMP)

The outer membrane proteins (OMP) were isolated as described by Achtman *et al.*, (1983). Cells were grown in 100 mL volumes of L-broth to mid-exponential phase ($4x10^8$ cells per mL) and suspended after centrifugation in 10 mL of 10 mM Tris buffer (pH 8.0). The cells were broken by sonication (80 s, 50% cycle, Branson B-30 cell disrupter), and unbroken cells were removed by centrifugation (Sorvall SS34 rotor, 3000 rpm, 20 min, 4°C). The resulting supernatant was centrifuged (Sorvall 50 Ti rotor, 20 000 rpm, 60 min, 4°C), and the pellet was suspended in 150 µL of distilled water and stored at -30°C. After thawing, 50 µL samples were extracted with 8 volumes of a detergent solution containing 1.67% (wt/vol) Sarkosyl and 11.1 mM Tris buffer (pH 7.6), each at a final concentration for 20 min at room temperature. The insoluble outer membranes were pelleted by centrifugation (Eppendorf microvials in adapters in a Sorvall SS34 rotor, 20 000 rpm, 90 min, 20°C) and suspended in 50 µL of electrophoresis sample buffer (0.0625 M Tris-HCl (pH 6.8); 10% (vol/vol) glycerol; 2% (wt/vol) SDS; 0.05% (vol/vol) 2-mercaptoethanol; 0.00125% (wt/vol) bromophenol blue and stored at -70°C.

2.15. SDS-PAGE for OMP

The OMPs were analysed by SDS-PAGE using the SDS-discontinous system of Laemmli (1970), using a Mini-Protean II system (Bio-Rad) apparatus. Proteins were stacked in a 4% acrylamide stacking gel and separated in a 12% acrylamide resolving gel containing 4 M urea. The buffer system was a Tris-glycine system consisting of 25 mM Tris-HCl , 192 mM glycine and 0.1% SDS (pH 8.3). After heat treatment of the samples, 30 µL of the original OM sample diluted 1:5 in sample buffer were loaded per lane and electrophoresis was carried out at a constant current of 30 mA per gel through the resolving gel. After completion of electrophoresis proteins were visualised by staining overnight with 0.1% (wt/vol) Coomassie blue in 45% (vol/vol) methanol/10% (vol/vol) acetic acid and by destaining with 30% (vol/vol) methanol/10% (vol/vol) acetic acid solution.

2.16. Reference Protein Markers

A molecular weight marker kit (Sigma MW-SDS-70) for SDS polyacrylamide gel electrophoresis (molecular weight range of 14000 to ca. 70000) was utilized as the reference marker. The reference marker possessed 6 proteins with the following sizes in kilodalton (kd); 14.3, 18.4, 24.0, 34.7, 45.0 and 66.0, respectively.

A standard curve was constructed for each acrylamide gel using the molecular weights of our standard marker and the distance migrated by each in the gel. Then the distance that each of the proteins extracted from the *S. enteritidis* isolates migrated within the same gel was measured and used to calculate the molecular weight of each protein in the isolates from the standard curve (Fig. 2.2).

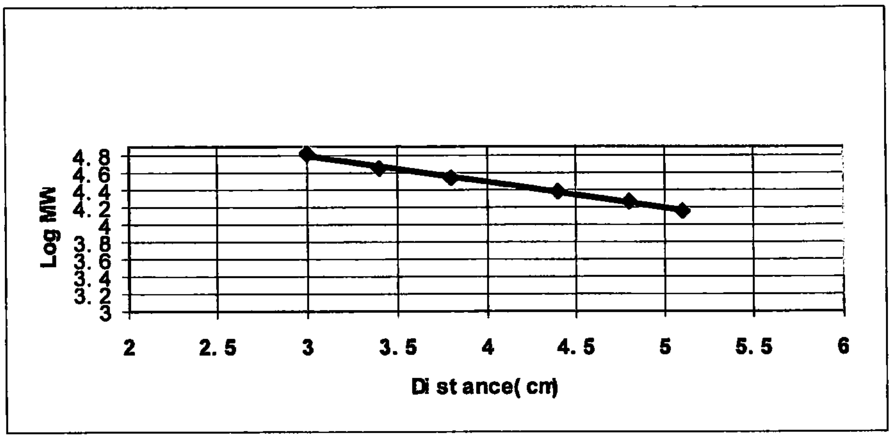


Figure 2.2. Standard curve for molecular weight estimation of OMPs

2.17. Phage Typing

Salmonella enteritidis strains and other isolates of the *Enterobacteriacea* were phagetyped by the Central Public Health Laboratory (CPHL) Laboratory of Enteric Pathogens, Colindale, United Kingdom. In this procedure the tested strains are grown over the entire plate. Plaques, or areas of lysis, are produced by bacteriophages, indicating that the strain is sensitive to infection by these phages.

2.18. Elimination of Plasmids

Curing of plasmid(s) was performed by incubation of the plasmid containing strain at 45°C for two to seven days (Poppe and Gyles, 1988).

2.19. Statistical Analysis of the Data

Principal Component Analysis method was used for the statistical analysis of the data. Principal component analysis (PCA) is a widely employed multivariate analysis technique that can reveal hidden groupings or splits in sets of taxa based on two or more sets of measurements (Quicke, 1993). The general principles are best illustrated for just two characters, but in practice there is no limit to the number of characters that can be included in PCA. The basic concept is that the variation in any one original variable (raw data) may not reveal the true diversity of the sample population. Thus as shown in Figure 2.3 a while both the x and y variables show some indication of heterogeneity, neither show the sample as comprising two discrete populations. However, brief inspection of the graph of x against y (the original variables) clearly shows that two populations are present. PCA is a method that identifies different axes (in this case other than x and y) that maximize the variance in the data as a whole. Thus in the figured example, two new axes, the first and second principal components, can be found and plotted such that plotting the data along the first principal component shows two clearly separated groups of data points (Figure 2.3b). Like the original variables x and y, the new variable axes are at right angles and are therefore independent of one another. While in this example the original data set comprises only two variables and simple visual inspection can easily reveal the inherent heterogeneity in the data, many situations are more complex, potentially involving many variables, and so plotting any two or three variables against one another may be inadequate to reveal underlying structure in the data. In this case PCA can be exteremly helpful (Quicke, 1993).

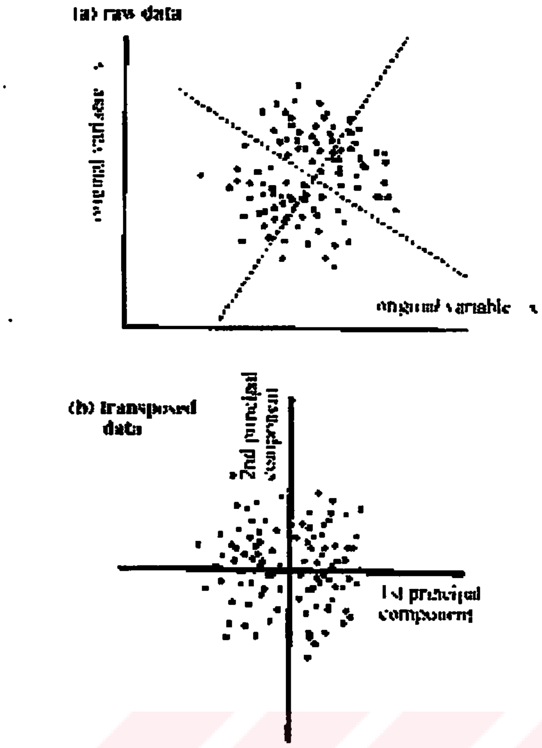


Figure 2.3. Principal components analysis of a two character data set.

(a)Data and principal components plotted against original variables;

(b) same data replotted with respect to the two principal components. Note that the distribution of data points along the first principal is distinctly bimodal whereas no bimodality is apparent in the distributions with respect to either of the original variables (from Quicke, 1993).

The actual PCA algorithms, which involve fairly complex matrix algebra and the identification of eigenvectors are too complex to describe but the principles are relatively easy to comprehend (Rholf, 1992). The aim of PCA is to discover a series of new vectors that are all at right angles to one another (i.e. they are orthogonal) such that the normalized data have maximum variance along the first principle component axis. The second principal component is then selected such that it is at right angles to the first and explains the maximum amount of the remaining variance in the data, i.e. that which is not explained by the first principal component. The third principal component is likewise at right angles to both the first and second and is selected so as to explain the maximum amount of variance in the data not yet explained by the first two principal components, and so on. In practice, the first two or three principal components usually explain most of the useful variation in the data and it is not common for workers to consider the fourth and higher components, although the total number of components that can be identified for a given data set is equal to the original number of characters. It should also be noted that the first principal component is often particularly strongly influenced by Operational Taxonomic Unit (OTU) size (even after normalization) because many characters display same degree of allometric growth. Therefore, if size is likely to be problematic, the second and third factors may be more informative from a taxonomic point of view. At the end of the PCA, data can be projected as in two or three dimensions using NTSYS-pc (Rholf, 1992). According to the similarities, dendogram is constructed using SIMGEND program in NTSYS-pc.

NTSYS-pc is a system of programs that is used to find and display structure in multivariate data. For example, one may wish to discover that a sample of data points represents samples from two or more distinct populations. Of equal interest is the discovery that some subsets of variables are highly intercorrelated. The program was originally developed for use in biology in the context of the field of numerical taxonomy (which explains why the name of the program is NTSYS- for Numerical

Taxonomy SYStem.). But the programs have also been widely used in morphometrics, ecology and in many other disciplines in the natural sciences, engineering, and the humanities. The terms mathematical taxonomy and automatic classification are also used sometimes to describe this field of application. The techniques also represent a subset of maltivariate data analysis and have close ties to some methods in the field of pattern recognition (Quicke, 1993).

CHAPTER 3

RESULTS

3.1. Antibiograms

3.1.1. Antibiotic Resistance Patterns of *S. enteritidis* Human Isolates

Thirty-three of fifty-one *S. enteritidis* human isolates were resistant to one or more of the antimicrobial agents employed in the drug testing panel (Table 3.1). Resistance to tetracycline was the most common resistance among 30 isolates, followed by resistance to cephalothin (10 isolates). Twenty of the all human isolates (39.21%) showed resistance to a single antibiotic. Thirteen of the human isolates (25.49%) showed multidrug-resistance. All the human isolates were sensitive to amikacin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, ofloxacin, tobramycin, cefodizime and meropehem.

Table 3.1. Antibiotic resistance patterns of *S. enteritidis* human isolates

Name of the Strain	Profile of the Resistance	Number of Strains	Number of Resistance Antibiotics	AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
H2, H3, H4, H6, H7, H17, H18, H19, H22, H25, H27, H30, H35, H37, H40, H44, H46, H47	I	18	0																									
H5	II	1	1																	R								
H8	III	1	1							R																		
H1,H9, H11, H12, H15, H20, H21, H26, H36, H39, H42, H48, H43, H45, H53, H58, H59, H63	IV	18	1																					R				
H10, H34, H41, H50	V	4	2																					R		R		
H13	VI	1	9		R	R		R	R	R	R									R			R	R				
H14	VII	1	9		R	R		R		R	R									R		R	R	R				
H16	VIII	1	8		R	R		R		R				R						R			R	R				
H24	IX	1	2							R														R				
H52	X	1	8			R			R	R				R		R					R			R		R		
H55	XI	1	2			R				R																		
H56	XII	1	3							R				R										R				
H57	XIII	1	4							R				R										R		R		
H62	XIV	1	7			R	R		R	R				R						R				R				
Antibiotics				AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
No. of Strains Investigated		51																										
Number of Strains Resistance to one Antibiotic		20 (39.21%)								1										1				18				
Number of Multiresistance Strains		13 (25.49%)			3	6	1	3	3	9	2			5		1				3	1	1	3	12		6		
Resistance Percentages of Antibiotics				0	5.88	11.76	1.96	5.88	5.88	19.6	3.92	0	0	9.8	0	1.96	0	0	0	7.84	1.96	1.96	5.88	58.82	0	11.76	0	0

AK	Amicasin-AK	CAZ	Ceftazidime-CAZ	PRL	Piperacillin-PRL	MP	Meropchem
AMC	Amoxicillin-AML+ Clavunalic Acid	CRD	Ceftriaxone-CRD	CFM	Cefixime-CFM		
AMP	Ampicillin	CXM	Cefuroxime-CXM	SP	Sulperazone-75 SP		
AZT	Aztreonam (Azactam)	CIP	Ciprofloxacin-CIP	SAM	Sulbactam Ampicillin-SAM		
CAR	Carbenicillin-Car	C	Chloramphenicol-c	TE	Tetracyclin-Te		
CEC	Cefaclor-CEC	GM	Gentamicin-GM-CN	TO	Tobramycin-NN		
CKF	Cephalothin-KF	IPM	Imipenem-IP	SXT	Trimethoprim Sulfamethoxazole		
CEP	Cefoperazone-CEF-CEP	OFX	Ofloxacin-OFX	CD	Cefodizime		

3.1.2. Statistical Analysis of the Antibiotic Resistance Patterns of *S. enteritidis* Human Isolates

The dendogram constructed by using SAHN clustering revealed two major classes of antibiotic resistance patterns among *S.enteritidis* human isolates (Figure 3.1)

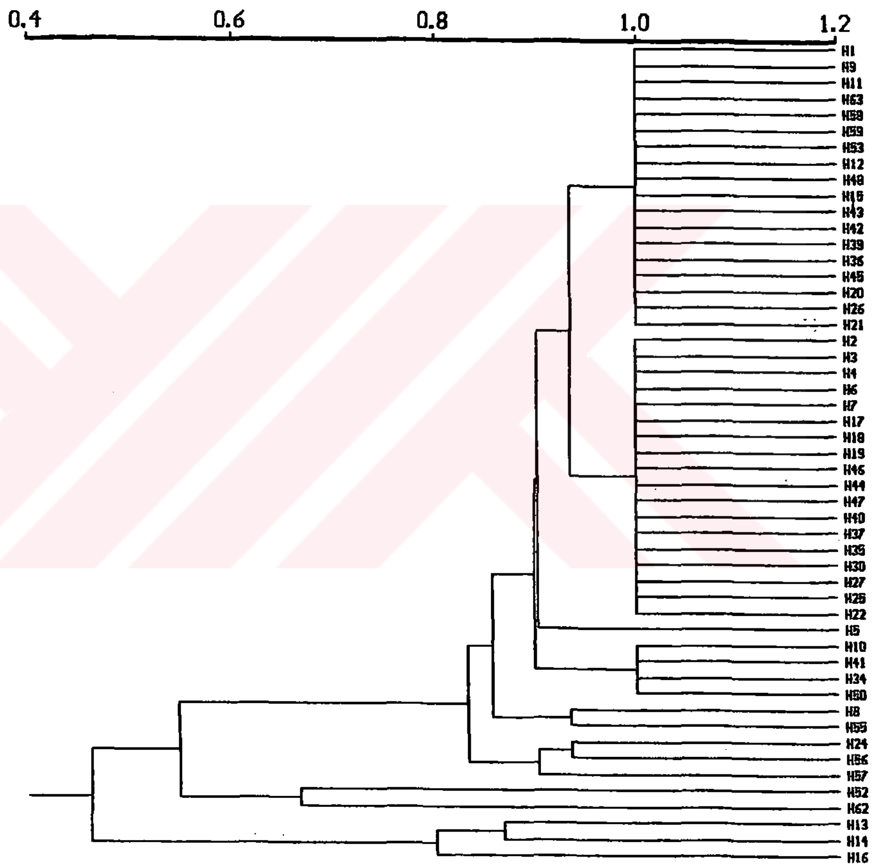


Figure 3.1. Dendogram constructed by using the antibiogram data of *S. enteritidis* human isolates

Principle Component Analysis (PCA) was also applied to the antibogram data of *S. enteritidis* human isolates (Figure 3.2). PCA resulted in two major clusters in human isolates of *S. enteritidis,* as was also evidenced by the dendogram shown in Figure 3.1. The isolates H62 and H52 would not be included in these two major clusters.

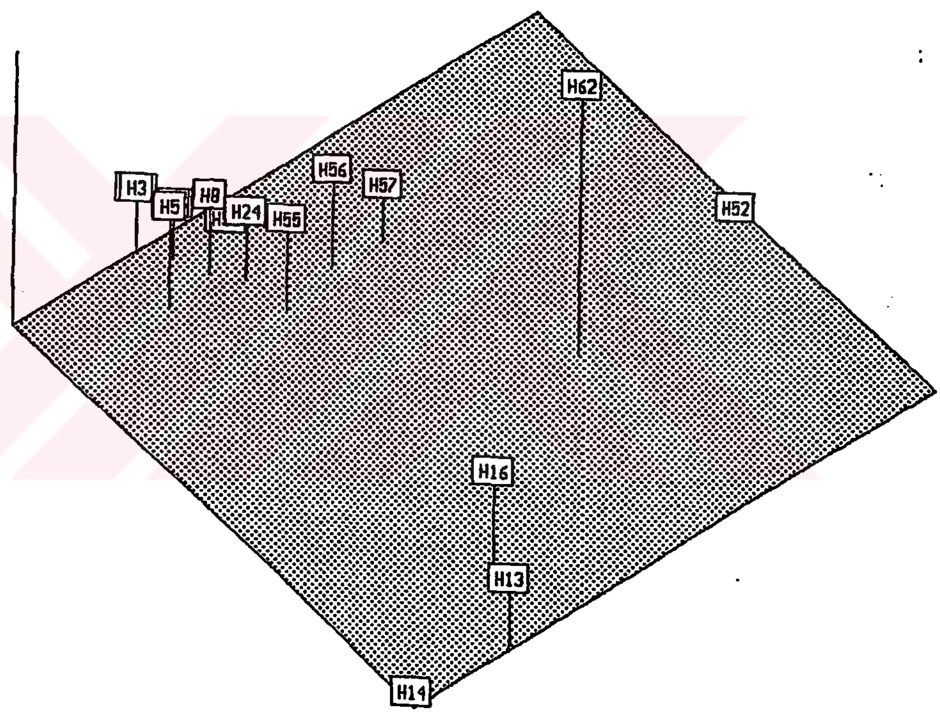


Figure 3.2. PCA of the antibiogram data of *S. enteritidis* human isolates in three dimensions

3.1.3. Antibiotic Resistance Patterns of *S. enteritidis* Chicken Isolates

Eighteen of twenty-one *S. enteritidis* chicken isolates were resistant to one or more of the antimicrobial agents tested (Table 3.2). Resistance to tetracycline was the most prevalent (15 isolates) which was followed by resistance to ampicillin (14 isolates) and resistance to carbenicillin (14 isolates). Four of these isolates (19.04%) showed drug resistance to a single antibiotic and the rest of the isolates (66.66%) showed multidrug-resistance. All the strains were sensitive to amikacin, aztreonam, cefaclor, cefoperazone, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, ofloxacin, cefixime, sulperazone, tobramycin, trimethoprim sulfamethoxone, cefodizime and meropehem.

Table 3.2. Antibiotic resistance patterns of *S. enteritidis* chicken isolates

Name of the Strain	Profile of the Resistance	Number of Strains	Number of Resistance Antibiotics	AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
A3, A22, A25, A26	IV	4	1																					R				
A9, A12, A23	I	3	0																									
A1, A16, A17	VIII	3	8		R	R		R		R				R						R			R	R				
A2, A14	XV	2	6			R		R		R				R						R				R				
A4	XVI	1	3			R		R																R				
A5	XVII	1	5			R		R		R				R										R				
A7, A13	XVIII	2	7			R		R		R				R						R			R	R				
A8	XIX	1	4			R		R		R														R				
A10	XX	1	4			R		R								R				R								
A11	XXI	1	4			R		R		R										R								
A15	XXII	1	5			R		R		R										R				R				
A19	XXIII	1	3			R				R										R								
Antibiotics				AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
No. of Strains Investigated		21																										
Number of Strains Resistance to one Antibiotic		4 (19.04%)																						4				
Number of Multiresistance Strains		14 (66.66%)			3	14	0	14		12				8		1				11			5	11				
Resistance Percentages of Antibiotics				0	14.28	66.66	0	53.84	0	57.14	0	0	0	38.09	0	4.76	0	0	0	52.38	0	0	23.8	71.42	0	0	0	0

AK	Amicasin-AK	CAZ	Ceftazidime-CAZ	PRL	Piperacillin-PRL	MP	Meropehem
AMC	Amoxicillin-AML+ Clavunalic Acid	CRD	Ceftriaxone-CRD	CFM	Cefixime-CFM		
AMP	Ampicillin	CXM	Cefuroxime-CXM	SP	Sulperazone-75 SP		
AZT	Aztreonam (Azactam)	CIP	Ciprofloxacin-CIP	SAM	Sulbactam Ampicillin-SAM		
CAR	Carbenicillin-Car	C	Chloramphenicol-c	TE	Tetracyclin-Te		
CEC	Cefaclor-CEC	GM	Gentamicin-GM-CN	TO	Tobramycin-NN		
CKF	Cephalothin-KF	IPM	Imipenem-IP	SXT	Trimethoprim Sulfamethoxazole		
CEP	Cefoperazone-CEF-CEP	OFX	Ofloxacin-OFX	CD	Cefodizime		

3.1.4. Statistical Analysis of the Antibiotic Resistance Patterns of *S. enteritidis* Chicken Isolates

The dendogram constructed by using SAHN clustering revealed four major clusters of antibiotic resistance patterns among *S. enteritidis* chicken isolates (Figure 3.3)

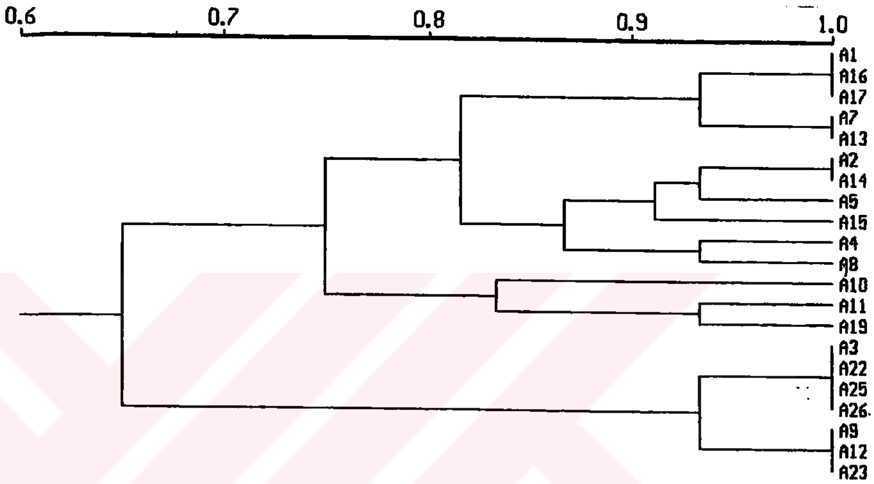


Figure 3.3. Dendoram constructed by using the antibiogram data of *S. enteritidis* chicken isolates

Principle Component Analysis (PCA) was also applied to the antibogram data of *S. enteritidis* chicken isolates (Figure 3.4) and revealed four major clusters in human isolates of *S. enteritidis* which accorded well to those obtained from the dendogram (Figure 3.3).

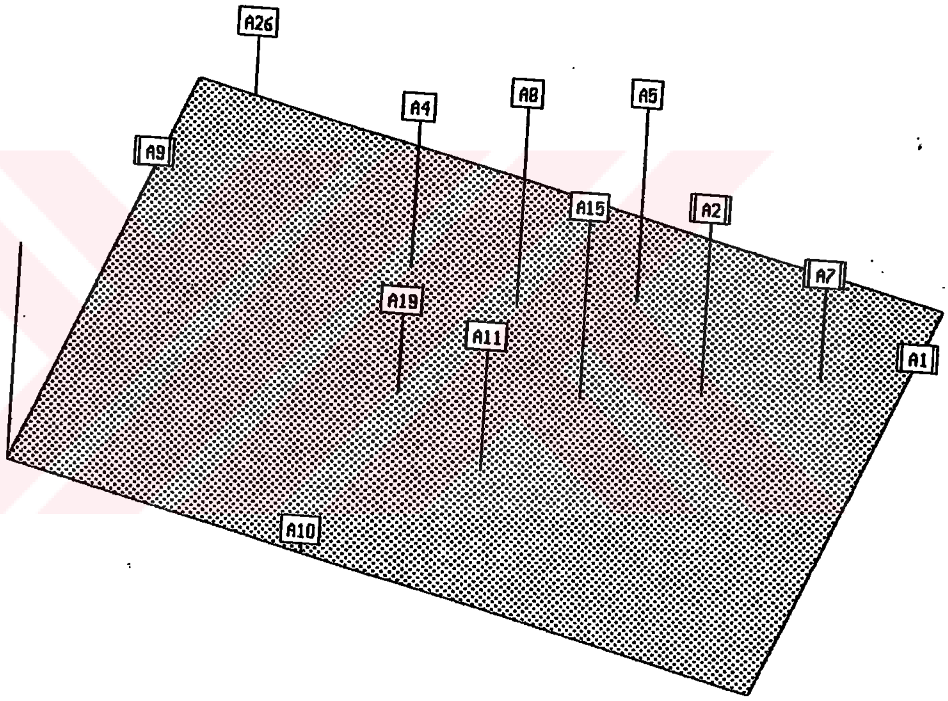


Figure 3.4. PCA of the antibiogram data of *S. enteritidis* chicken isolates in three dimensions

3.1.5. Antibiotic Resistance Patterns of *S. enteritidis* Egg Isolates

Five out of ten *S. enteritidis* chicken isolates were resistant to one or more of the antimicrobial agents tested (Table 3.3). Resistance to tetracycline was most common (7 isolates). Five of these isolates (50%) showed drug resistance to only one antibiotic. Two of the egg isolates (20%) showed multidrug-resistance. All the isolates were sensitive to amikacin, amoxicillin, aztreonam, carbenicillin, cefaclor, cefoperazone, ceftazidime, ceftriaxone, ciprofloxacin, chloramphenicol, gentamicin, imipenem, ofloxacin, piperacillin, cefixime, sulperazone, sulbactam ampicillin, tobramycin, trimethoprim sulfamethoxone, cefodizime and meropehem.

Table 3.3. Antibiotic resistance patterns of *S. enteritidis* egg isolates

Name of the Strain	Profile of the Resistance	Number of Strains	Number of Resistance Antibiotics	ANTIBIOTICS																								
				AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
E1, E3, E4, E7, E11	IV	5	1																					R				
E9, E10, E13	I	3	0																									
E8, E12	XXIV	2	4			R				R				R										R				
Antibiotics				AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
No. of Strains Investigated		10																										
Number of Strains Resistance to one Antibiotic		5 (50.00%)																						5				
Number of Multiresistance Strains		2 (20.00%)				2				2				2										2				
Resistance Percentages of Antibiotics				0	0	20	0	0	0	20	0	0	0	20	0	0	0	0	0	0	0	0	0	70	0	0	0	0

AK	Amicasin-AK	CAZ	Ceftazidime-CAZ	PRL	Piperacillin-PRL	MP	Meropehem
AMC	Amoxicillin-AML+ Clavunalic Acid	CRD	Ceftriaxone-CRD	CFM	Cefixime-CFM		
AMP	Ampicillin	CXM	Cefuroxime-CXM	SP	Sulperazone-75 SP		
AZT	Aztreonam (Azactam)	CIP	Ciprofloxacin-CIP	SAM	Sulbactam Ampicillin-SAM		
CAR	Carbenicillin-Car	C	Chloramphenicol-c	TE	Tetracyclin-Te		
CEC	Cefaclor-CEC	GM	Gentamicin-GM-CN	TO	Tobramycin-NN		
CKF	Cephalothin-KF	IPM	Imipenem-IP	SXT	Trimethoprim Sulfamethoxazole		
CEP	Cefoperazone-CEF-CEP	OFX	Ofloxacin-OFX	CD	Cefodizime		

3.1.6. Statistical Analysis of the Antibiotic resistance Patterns of *S. enteritidis* Egg Isolates

When a dendogram was constructed using the antibiotic resistance patterns of *S. enteritidis* egg isolates, three major clusters were obtained (Figure 3.5). In the first cluster E1, E3, E4, E7 and E11, in the second cluster E9, E13 and E10, and in the third cluster E8 and E12 showed a high percentage of similarity, respectively.

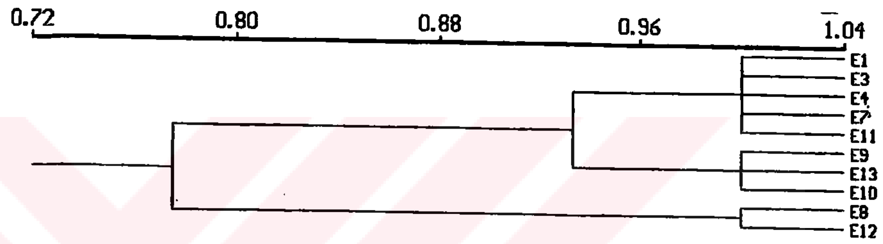


Figure 3.5. Dendoram constructed by using the antibiogram data of *S. enteritidis* egg isolates

3.1.7. A General Outlook to the Antibiotic Resistance Patterns of *S. enteritidis* Isolates of Different Sources

It was observed that none of the *S. enteritidis* strains from human, chicken and egg sources showed resistance to aminoglycoside, quinolone and carbapenem type antibiotics (Table 3.4). On the other hand, it was recorded that *S. enteritidis* strains from all these sources have started to develop resistance to cephems of class II and III types. Resistance to tetracycline was the most common among *S. enteritidis* strains of the different sources. Although resistance to sulfamide was prevalent among human isolates, this kind of resistance has not been observed from the strains isolated from chicken or egg. *S. enteritidis* strains isolated from human and chicken sources showed resistance to chloramphenicol and β lactam/β lactamase inhibitor combinations, whereas the strains isolated from egg were completely sensitive to these types of antibiotics.

Table 3.4. A general outlook to the antibiotic resistance patterns of *S. enteritidis* isolates of different sources

Source of the Strains	Number of Profile of the Resistance	Number of Strains	Number of Strains Resistance to one Antibiotic	Number of Multiresistance Strains	Antibiotics																								
					Aminoglycosides			b Lactams			Cephems (Cephalospors)								Sulfonamides	b lactam/ b Lactamase Inhibitor Combinations			Quinolones		Monobactams	Carbapenems		Tetracyclines	Chloramphenicol
											I	II		III															
					AK	GM	TO	AMP	PRL	CAR	CKF	CEC	CXM	CEP	CAZ	CRD	CFM	CD	SXT	SAM	AMC	SP	CIP	OFX	AZT	IPM	MP	TE	C
Human	14	51	20 (39.21%)	13 (25.49%)	0	0	0	11.76	7.84	5.88	19.6	5.88	9.8	3.92	0	0	1.96	0	11.76	5.88	5.88	1.96	0	0	1.96	0	0	58.82	1.96
Chicken	12	21	4 (19.04%)	14 (66.66%)	0	0	0	66.66	52.38	53.8	57.14	0	38.09	0	0	0	0	0	0	23.8	14.28	0	0	0	0	0	0	71.42	4.76
Egg	3	10	5 (50.00%)	2 (20.00%)	0	0	0	20	0	0	20	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	70	0

AK Amicasin-AK
AMC Amoxicillin-AML+ Clavunalic Acid
AMP Ampicillin
AZT Aztreonam (Azactam)
CAR Carbenicillin-Car
CEC Cefaclor-CEC
CKF Cephalothin-KF
CEP Cefoperazone-CEF-CEP
CAZ Ceftazidime-CAZ
CRD Ceftriaxone-CRD
CXM Cefuroxime-CXM
CIP Ciprofloxacin-CIP
C Chloramphenicol-c
GM Gentamicin-GM-CN
IPM Imipenem-IP
OFX Ofloxacin-OFX
PRL Piperacillin-PRL
CFM Cefixime-CFM
SP Sulperazone-75 SP
SAM Sulbactam Ampicillin-SAM
TE Tetracyclin-Te
TO Tobramycin-NN
SXT Trimethoprim Sulfamethoxazole
CD Cefodizime
MP Meropehem

3.1.8. Statistical Analysis of the Antibiotic Resistance Patterns of *S. enteritidis* Isolates As a Whole

When dendogram was constructed by using the antibiogram data from the isolates of different sources, eight major clastures were obtained (Figure 3.6). H62 and H52 isolates were again out of the clusters obtained. On the other hand chicken isolates designated A25, A22, A26, A3, A23, A12, A9 and egg isolates designated E11, E7, E3, E1, E4, E13, E9, E10 displayed a high percentage of similarity to the human isolates.

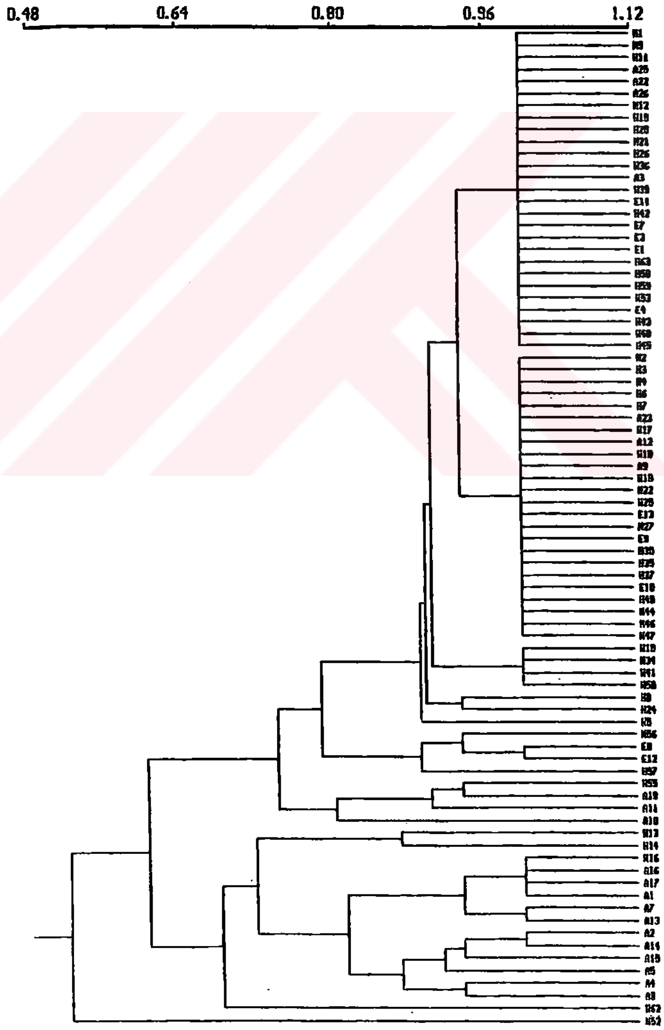


Figure 3.6. Dendogram constructed by using the antibiotic resistance patterns of *S.enteritidis* isolates of human, chicken and egg origin

Principle Component Analysis (PCA) was next applied to the antibogram data of *S. enteritidis* of different origin (Figure 3.7). It was the evident that the isolates H62 and H52 were out of the two major clusters obtained.

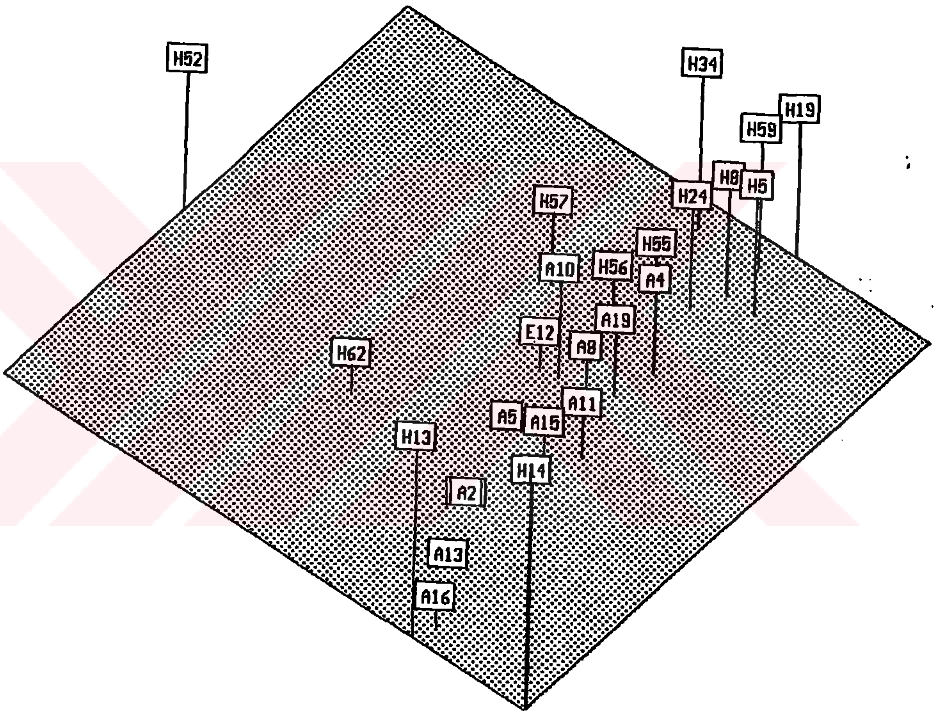


Figure 3.7. PCA of antibiogram results of *S. enteritidis* strains in three dimensions

3.1.9. Antibiotic Resistance Patterns of Other Members of the Family *Enterobacteriaceae*

Besides the *S. enteritidis* isolates of human, chicken and egg origin mentioned in previous sections of this Chapter, twelve different members of the family *Enterobacteriaceae* were also tested for their antibiotic resistance. It was found that none of these isolates showed resistance to aminoglycoside, quinolone and carbapenem type antibiotics (Table 3.5). As observed with *S. enteritidis* strains, other enterics have also started to develop resistance to cephem class II and III types of antibiotics.Tetracycline resistance was also the most common (80%) among these enterics. Of twelve different antibiotic profiles obtained from these isolates only two of the profiles (profiles I and II) were shared with *S. enteritidis* isolates. 50% of these strains were multiresistant.

Table 3.5. Antibiotic resistance patterns of other enterics used in the study

				Antibiotics																								
				Aminoglycosides			b Lactams			Cephems (Cephalospors)								Sulfonamides	B lactam/ b lactamase Inhibitor Combinations			Quinolones		Monobactams	Carbapenems		Tetracyclines	Chloramphenicol
										I	II		III															
Name of the Strain	Profile of the Resistance	Number of Strains	Number of Resistance Antibiotics	AK	GM	TO	AMP	PRL	CAR	CKF	CEC	CXM	CEP	CAZ	CRD	CFM	CD	SXT	SAM	AMC	SP	CIP	OFX	AZT	IPM	MP	TE	C
H28, A18	II	2	0																									
H33, H38, H54, E2, E5, E6, A6, A21	I	8	1																								R	
H23	XXV	1	3					R				R															R	
H29	XXVI	1	9				R	R		R			R					R	R					R			R	R
H31	XXVII	1	2																								R	R
H32	XXVIII	1	3				R	R	R																			
H49	XXIX	1	9				R	R	R	R		R							R	R							R	R
H51	XXX	1	11				R	R	R	R	R	R				R			R	R							R	R
H60	XXXI	1	4				R			R																	R	R
H61	XXXII	1	7				R			R	R	R							R	R							R	
A20	XXXIII	1	5				R	R	R			R												R				
A24	XXXIV	1	2																					R			R	
Antibiotics				AK	GM	TO	AMP	PRL	CAR	CKF	CEC	CXM	CEP	CAZ	CRD	CFM	CD	SXT	SAM	AMC	SP	CIP	OFX	AZT	IPM	MP	TE	C
No. of Strains Investigated		20																										
Number of Strains Resistance to one Antibiotic		8(40%)																									8	
Number of Multiresistance Strains		10(50%)					7	6	4	5	2	5	1			1		1	4	3				3			8	5
Resistance Percentages of Antibiotics				0	0	0	35	30	20	25	10	25	5	0	0	5	0	5	20	15	0	0	0	15	0	0	80	25

AK	Amicasin-AK	CAZ	Ceftazidime-CAZ	PRL	Piperacillin-PRL	MP	Meropehem
AMC	Amoxicillin-AML+ Clavunalic Acid	CRD	Ceftriaxone-CRD	CFM	Cefixime-CFM		
AMP	Ampicillin	CXM	Cefuroxime-CXM	SP	Sulperazone-75 SP		
AZT	Aztreonam (Azactam)	CIP	Ciprofloxacin-CIP	SAM	Sulbactam Ampicillin-SAM		
CAR	Carbenicillin-Car	C	Chloramphenicol-c	TE	Tetracyclin-Te		
CEC	Cefaclor-CEC	GM	Gentamicin-GM-CN	TO	Tobramycin-NN		
CKF	Cephalothin-KF	IPM	Imipenem-IP	SXT	Trimethoprim Sulfamethoxazole		
CEP	Cefoperazone-CEF-CEP	OFX	Ofloxacin-OFX	CD	Cefodizime		

3.2. Localization of Chromosomal DNA

In order to define the localization of the chromosomal DNA of the *S. enteritidis* isolates against the marker and hence to prevent a confusion in plasmid analyses, chromosomal DNA was isolated from *S. enteritidis* chicken isolates (A11, A12, A15 and A16) and human isolates (H4, H5 and H6). The results are presented in Figure 3.8.

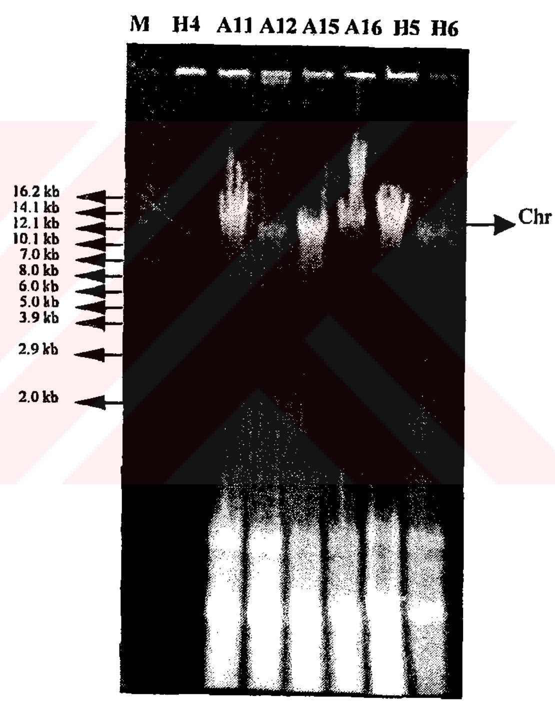


Figure 3.8. Chromosomal DNA from various *S. enteritidis* isolates (M Marker)

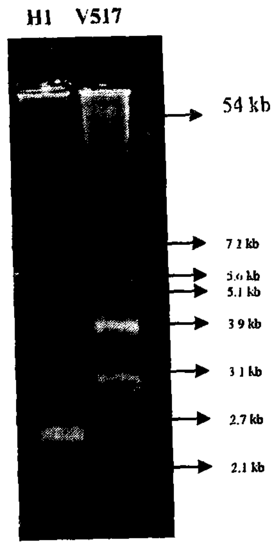
3.3. Plasmid Patterns

3.3.1. Plasmid Patterns of *S. enteritidis* Human Isolates

The plasmid profiles of the human isolates are shown in Figure 3.9 and summarized in Table 3.6. The fifty-one human isolates examined demonstrated 13 different plasmid profiles. The plasmid sizes ranged between 54 and 0.9 kb. It was found that high molecular weight plasmid of 54 kb was harbored by 94.11% of the human isolates. The plasmid number varied between zero (isolate H52) and to ten (isolate H55).

Table 3.6. Plasmid profiles of *S. enteritidis* human isolates

Strain Designation	Source	Profile of the Plasmid	Number of Strains	Number of Plasmids	Plasmid Profiles in kb									
H2, H4, H5, H6, H7, H9, H11, H21	Human	I	8	3	54	2.4	1.9							
H1, H3	Human	II	2	2	2.4	1.9								
H8	Human	III	1	4	54	4.4	2.4	1.4						
H10, H12, H13, H14, H17, H18, H19, H20, H24, H25, H26, H27, H30, H34, H35, H36, H39, H41, H42, H43, H44, H45, H46, H47, H48, H50, H57, H58, H62, H63	Human	IV	30	1	54									
H15	Human	V	1	5	54	4.4	2.6	1.9	1.2					
H16	Human	VI	1	2	54	33.8								
H22	Human	VII	1	2	54	23								
H52	Human	VIII	1	0										
H37	Human	IX	1	3	54	4.8	3.9							
H40, H56	Human	X	2	2	54	3.2								
H53	Human	XI	1	3	54	27.5	9.5							
H55	Human	XII	1	10	54	27.5	13	9.5	5	4.1	2.1	1.6	1.3	0.9
H59	Human	XIII	1	5	54	4.1	2.1	1.6	0.9					



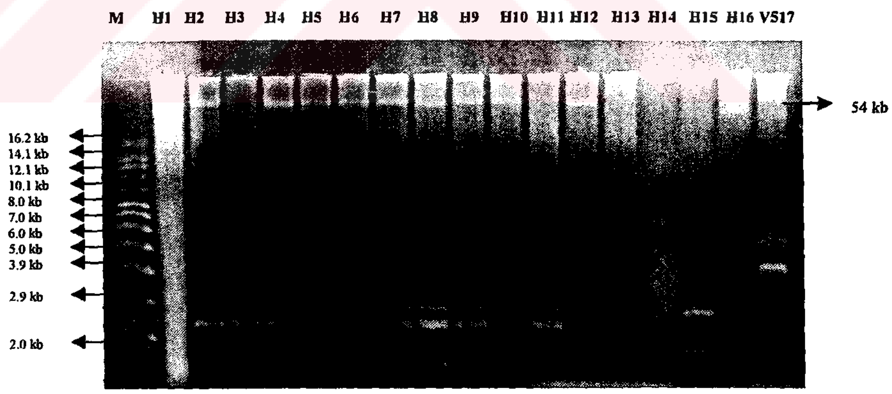
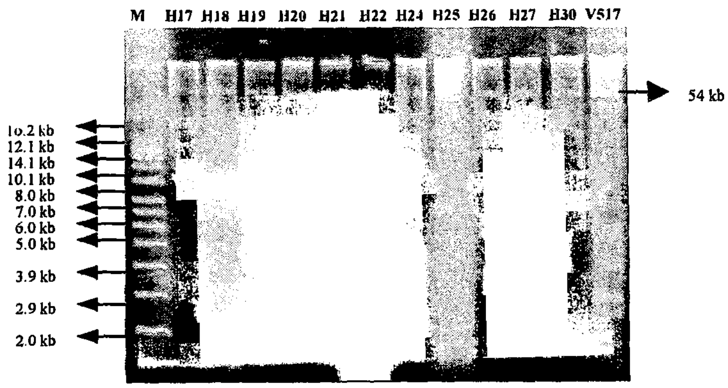
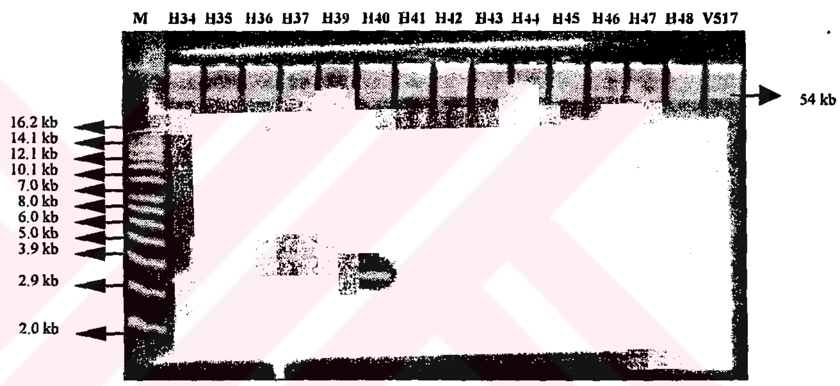


Figure 3.9. Plasmid profiles of *S. enteritidis* human isolates





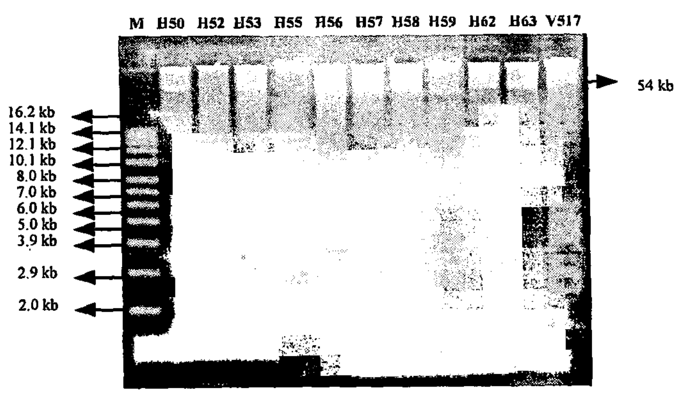


Figure 3.9. (Cont'd)

3.3.2. Statistical Analysis of the Plasmid Profiles of *S. enteritidis* Human Isolates

A dendogram was constructed to make a statistical analysis of plasmid data. Two main clusters were obtained (Figure 3.10). The isolates H55, H59, H15, H53 and H8 were out of the clusters.

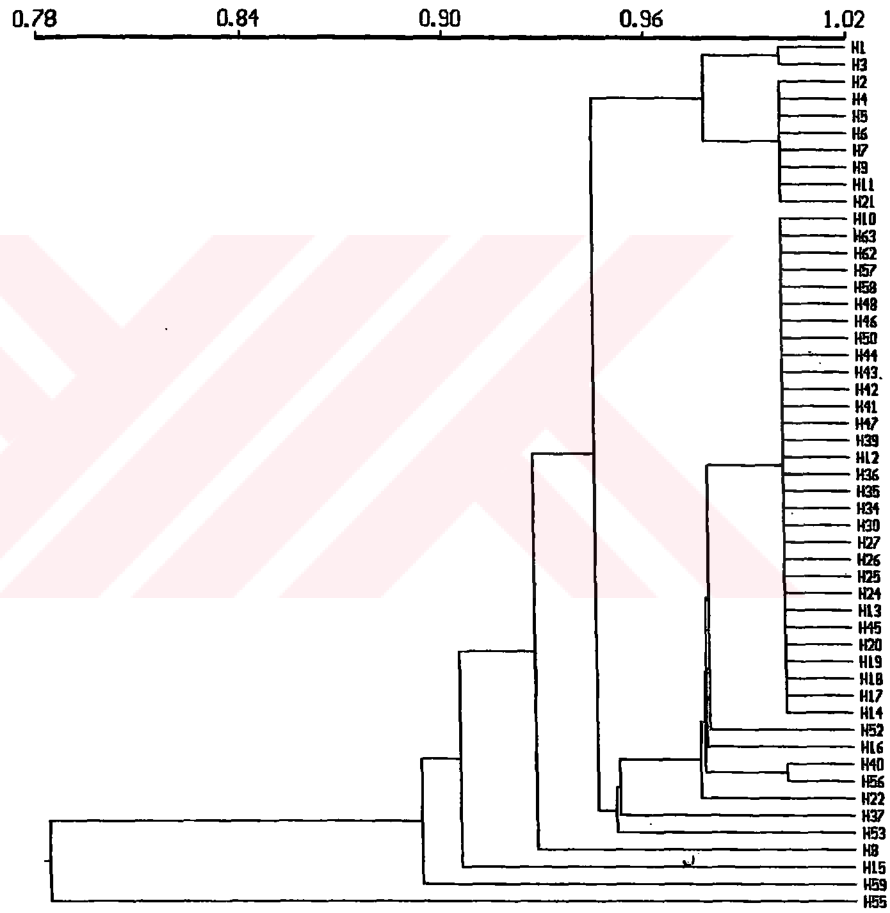


Figure 3.10. Dendogram constructed for statistical analysis of the plasmid profiles of *S. enteritidis* human isolates

PCA was also applied to the plasmid profile data of *S. enteritidis* human isolates (Figure 3.11).

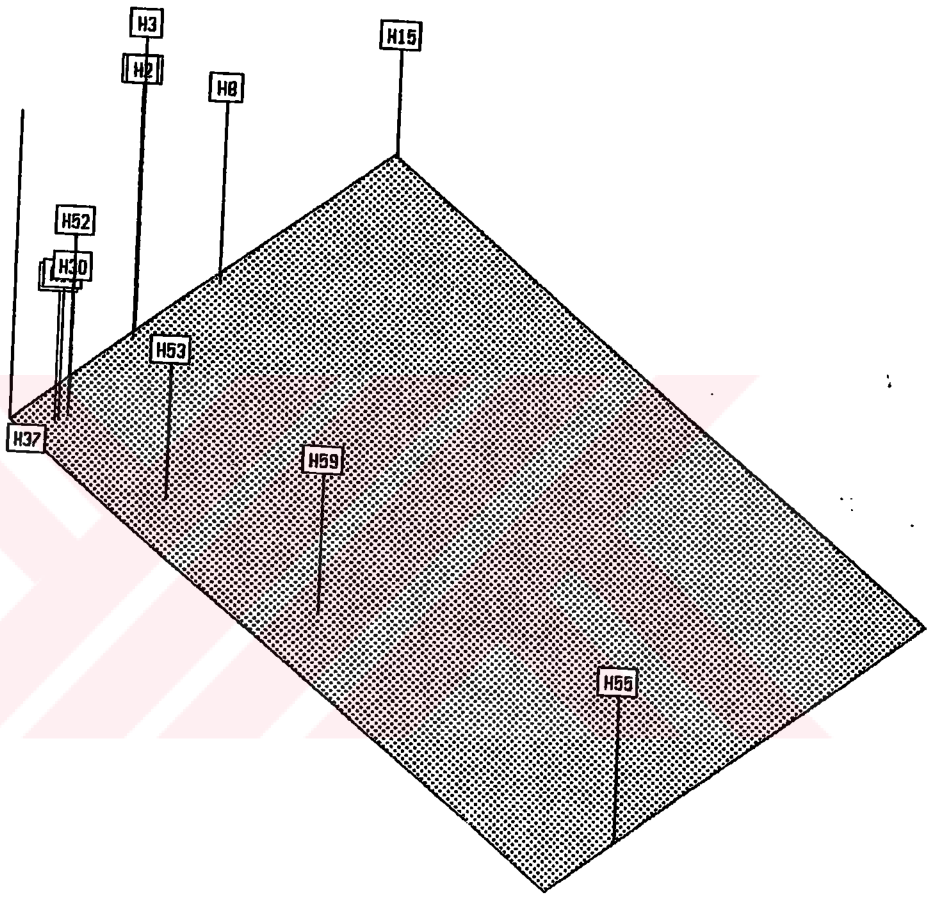


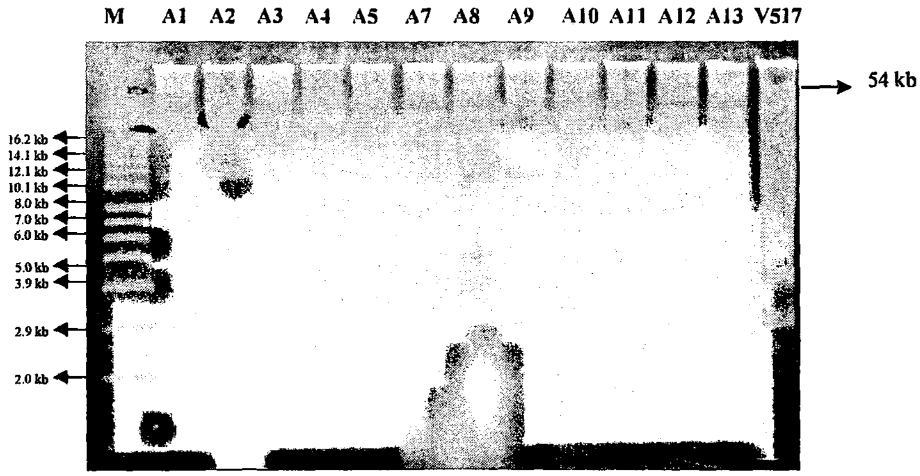
Figure 3.11. PCA of the plasmid profiles of *S. enteritidis* human isolates in three dimensions

3.3.3. Plasmid Patterns of *S. enteritidis* Chicken Isolates

The plasmid profiles of the chicken isolates are shown in Figure 3.12 and summarized in Table 3.7. The twenty-one chicken isolates gave 5 different plasmid profiles. The plasmid sizes ranged between 54 to 22.9 kb. The plasmid content ranged from one plasmid in isolates A3, A4, A14, A15, A16, A17 and A19 to 2 plasmids in isolates A1, A2, A7, A8, A9, A5, A10, A11, A12, A13, A22, A23, A25 and A26. The 54 kb high molecular weight plasmid was contained by 90.47% of the chicken isolates.

Table 3.7. Plasmid profiles of *S. enteritidis* chicken isolates

Strain Designation	Profile of the Plasmid	Number of Strains	Number of Plasmids	Plasmid Profiles in kb	
A1, A2, A7, A8, A9	XIV	5	2	54	22.9
A3, A4, A14, A15, A16	IV	5	1	54	
A5, A10, A11, A12, A13	XV	5	2	54	28.7
A17, A19	XVI	2	1	31.9	
A22, A23, A25, A26	XVII	4	2	54	45.5



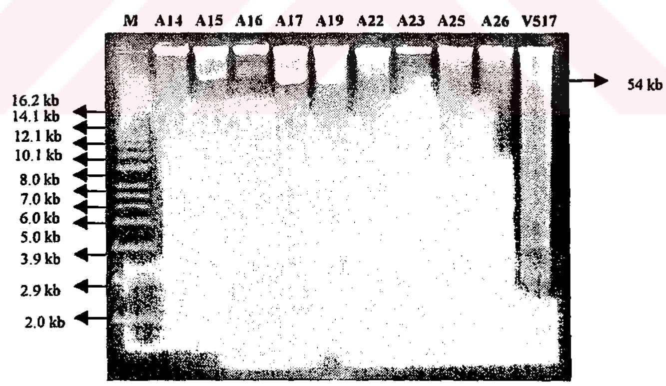


Figure 3.12. Plasmid profiles of *S. enteritidis* chicken isolates

3.3.4. Statistical Analysis of the Plasmid Profiles of *S. enteritidis* Chicken Isolates

When dendogram was constructed by SAHN clustering, five major clusters were obtained (Figure 3.13).

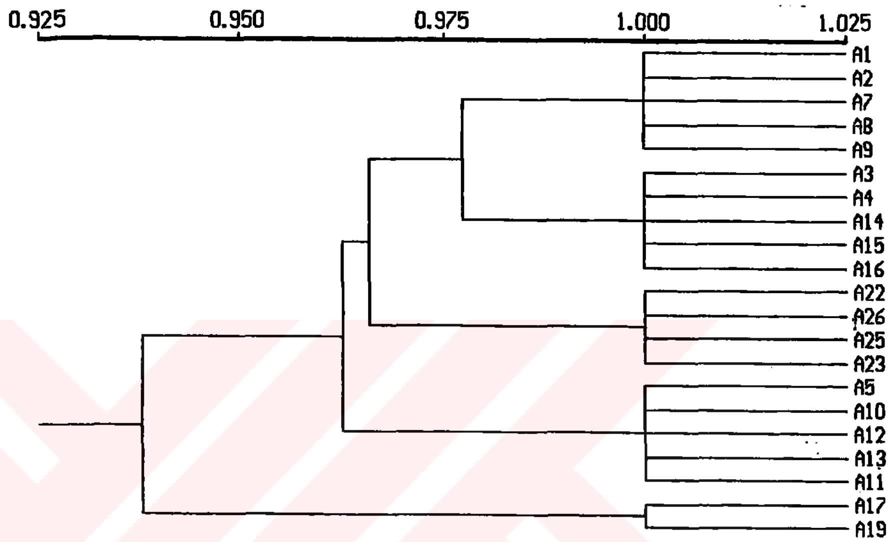


Figure 3.13. Dendogram constructed for statistical analysis of the plasmid profiles of *S. enteritidis* chicken isolates

The clustering obtained by PCA is shown in Figure 3.14.

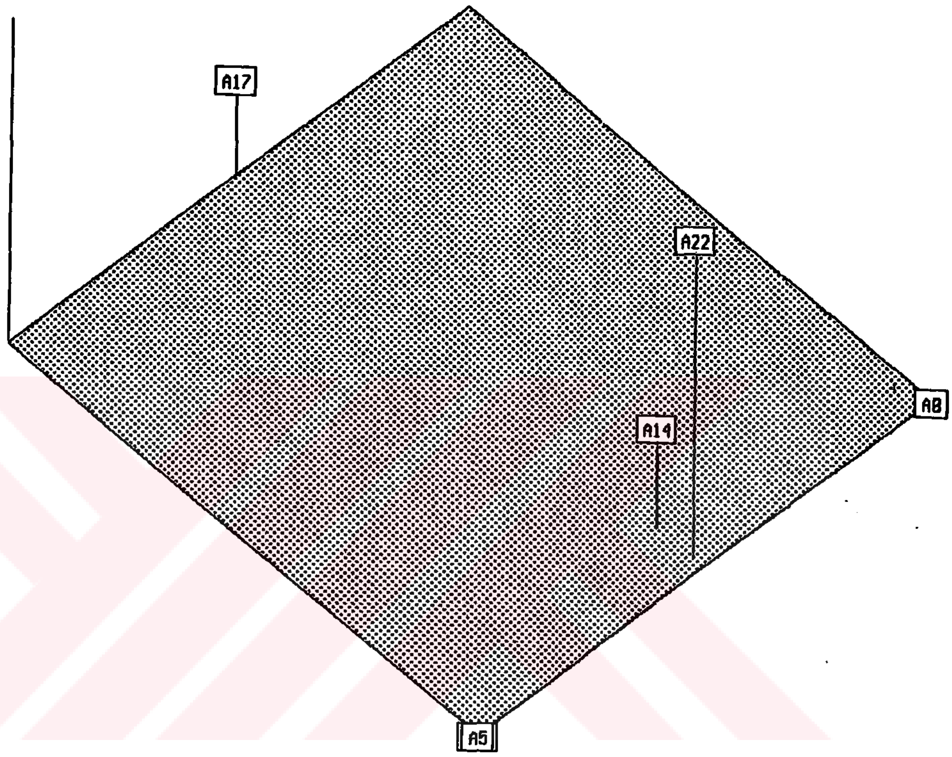


Figure 3.14. PCA of the plasmid profiles of *S. enteritidis* chicken isolates in three dimensions

3.3.5. Plasmid Patterns of *S. enteritidis* Egg Isolates

The plasmid profiles of the egg isolates are shown in Figure 3.15 and summarized in Table 3.8. The ten egg isolates displayed six different plasmid profiles. The plasmid sizes ranged between 54 to 1.6 kb. The plasmid numbers ranged from one plasmid in isolate E3 to up to five plasmids in isolate E11. The 54 kb plasmid was harbored by all of the egg isolates.

Table 3.8. Plasmid profiles of *S. enteritidis* egg isolates

Strain Designation	Profile of the Plasmid	Number of Strains	Number of Plasmids	Plasmid Profiles in kb				
E1, E4, E8, E10, E12	XVIII	5	3	54	29.7	16.5		
E3	IV	1	1	54				
E7	XIX	1	4	54	16.5	2.2	1.6	
E9	XX	1	2	54	29.7			
E11	XXI	1	5	54	29.7	16.5	5.7	4.5
E13	XXII	1	2	54	16.5			

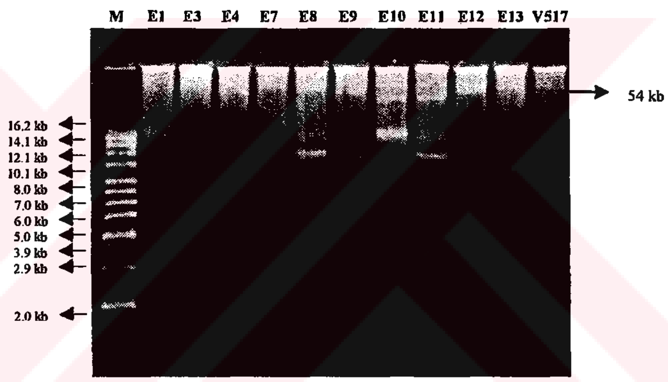


Figure 3.15. Plasmid profiles of *S. enteritidis* egg isolates

3.3.6. Statistical Analysis of the Plasmid Profiles of *S.enteritidis* Egg Isolates

The dendogram gave rise to two main clusters as shown in Figure 3.16. The isolates E7 and E11 were found to be out of the clusters.

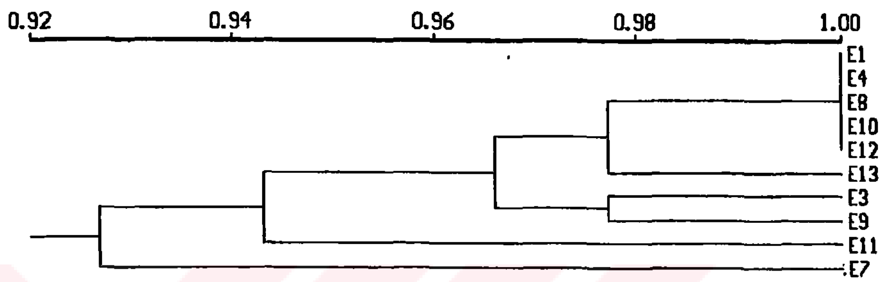


Figure 3.16. Dendogram constructed for the statistical analysis of the plasmid data of *S. enteritidis* egg isolates

PCA was applied to the plasmid profile data of *S. enteritidis* egg isolates (Figure 3.17). PCA clustering like the dendogram, revealed that the isolates E7 and E11 were out of the clusters.

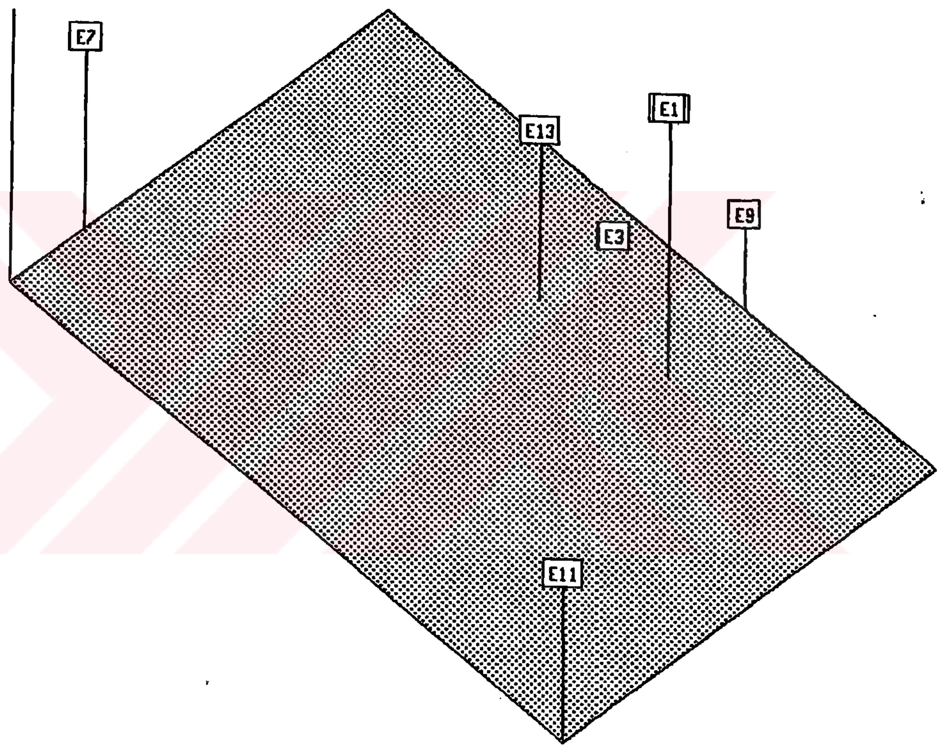


Figure 3.17. PCA of the plasmid profiles of *S. enteritidis* egg isolates in three dimensions

3.3.7. A Comparison Between the Plasmid Patterns of *S. enteritidis* Strains of Different Origin

Table 3.9 represents a comparison between isolates from different sources. The human isolates revealed the greatest variation in plasmid numbers. Chicken isolates varied less in plasmid numbers as they harbored either one or two plasmids. The majority of human isolates possessed at least one plasmid. All of the egg isolates had the 54 kb high molecular weight plasmid which was harbored by 94.11% of the human isolates and 90.47% of the chicken isolates.

Table 3.9. Summary of plasmid profiles of *S. enteritidis* strains

Sources of Isolates	Number of Isolates	Number of Different Plasmid Profiles	Number of Plasmids / Number of Isolates											% of Isolates with 54 kb Plasmid
			0	1	2	3	4	5	6	7	8	9	10	
Human	51	13	1	30	6	10	1	2	0	0	0	0	1	94.11
Chicken	21	5	0	7	14	0	0	0	0	0	0	0	0	90.47
Egg	10	6	0	1	2	5	1	1	0	0	0	0	0	100.00

3.3.8. Statistical Analysis of Plasmid Profiles of *S. enteritidis* Strains of Different Origin

When a dendogram was constructed, 6 main clusters were obtained from the plasmid profiles of *S. enteritidis* isolates from three different sources (Figure 3.18). H55, H59, H15, E11, E7, H8, H53 and H37 were out of clusters. The chicken isolates A16, A14, A15, A4 and A3 showed high percentage of similarity with human isolates.

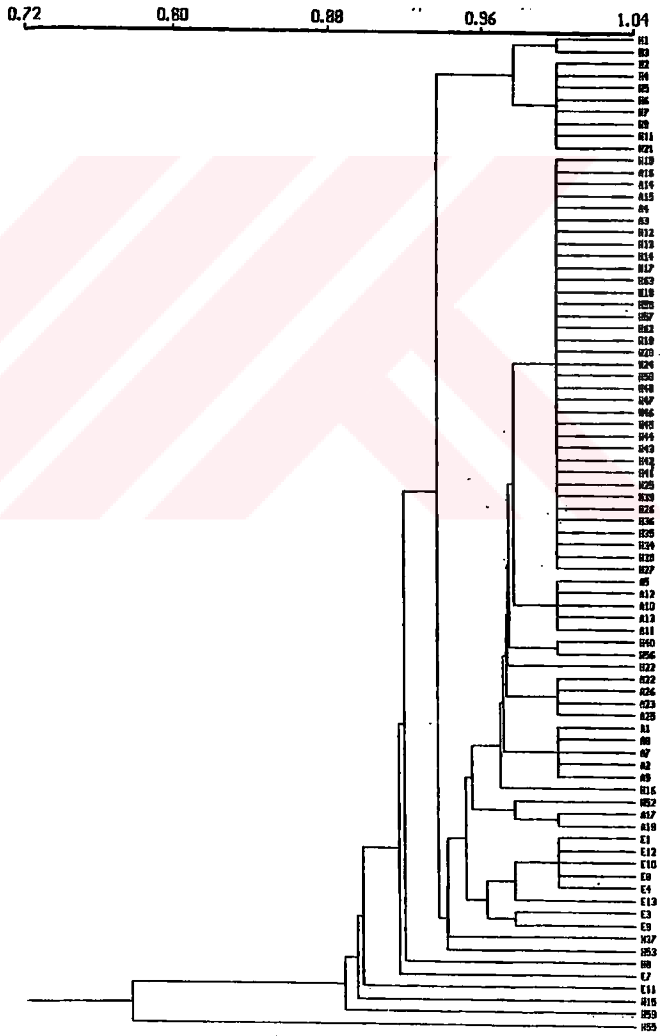


Figure 3.18. Dendogram constructed from the plasmid profiles of *S. enteritidis* strains

PCA clustering is shown in Figure 3.19.

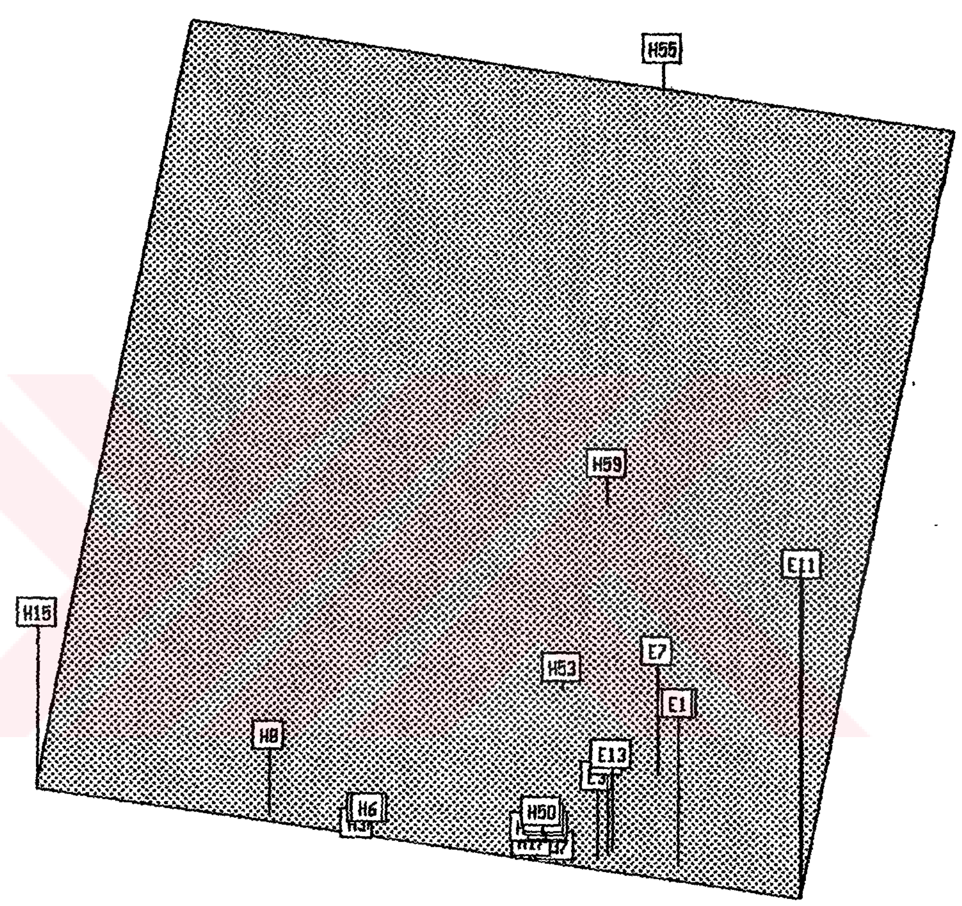


Figure 3.19. PCA of plasmid profiles of *S. enteritidis* strains in three dimensions.

3.3.9. Plasmid Profiles of Other Species of *Enterobacteriaceae*

The plasmid profiles of the other enterics used in this study are shown in Figure 3.20 and summarized in Table 3.10. Twenty isolates displayed 13 different plasmid profiles. The plasmid sizes ranged between 54 to 1.2 kb. The plasmid numbers ranged from zero (isolates H23, H51, H60 and A21) to seven plasmids (isolate H28). The 54 kb high molecular weight plasmid was harbored by 55% of these enteric species.

Table 3.10. Plasmid profiles of other enterics

Strain Designation	Profile of the Plasmid	Number of Strains	Number of Plasmids	Plasmid Profiles in kb									
E5, E6	XVIII	2	3	54	29.7	16.5							
E2	XXIII	1	1	1.6									
H29, H31, H61	IV	3	1	54									
H28	XXIV	1	7	54	28.4	5.3	4.3	3.7	3.5	1.5			
H32	XXV	1	4	54	28.4	2.3	1.9						
H33	XXVI	1	4	54	4.8	2.3	2.1						
H23, H51, H60, A21	VIII	4	0										
H38	XXVII	1	1	24.9									
H49	XXVIII	1	3	54	3.3	2.8							
H54	XI	1	3	54	27.5	9.5							
A6, A20	XXIX	2	4	5.5	4.9	3.9	3.5						
A18	XXX	1	4	2.5	1.8	1.4	1.2						
A24	XVII	1	2	54	45.5								

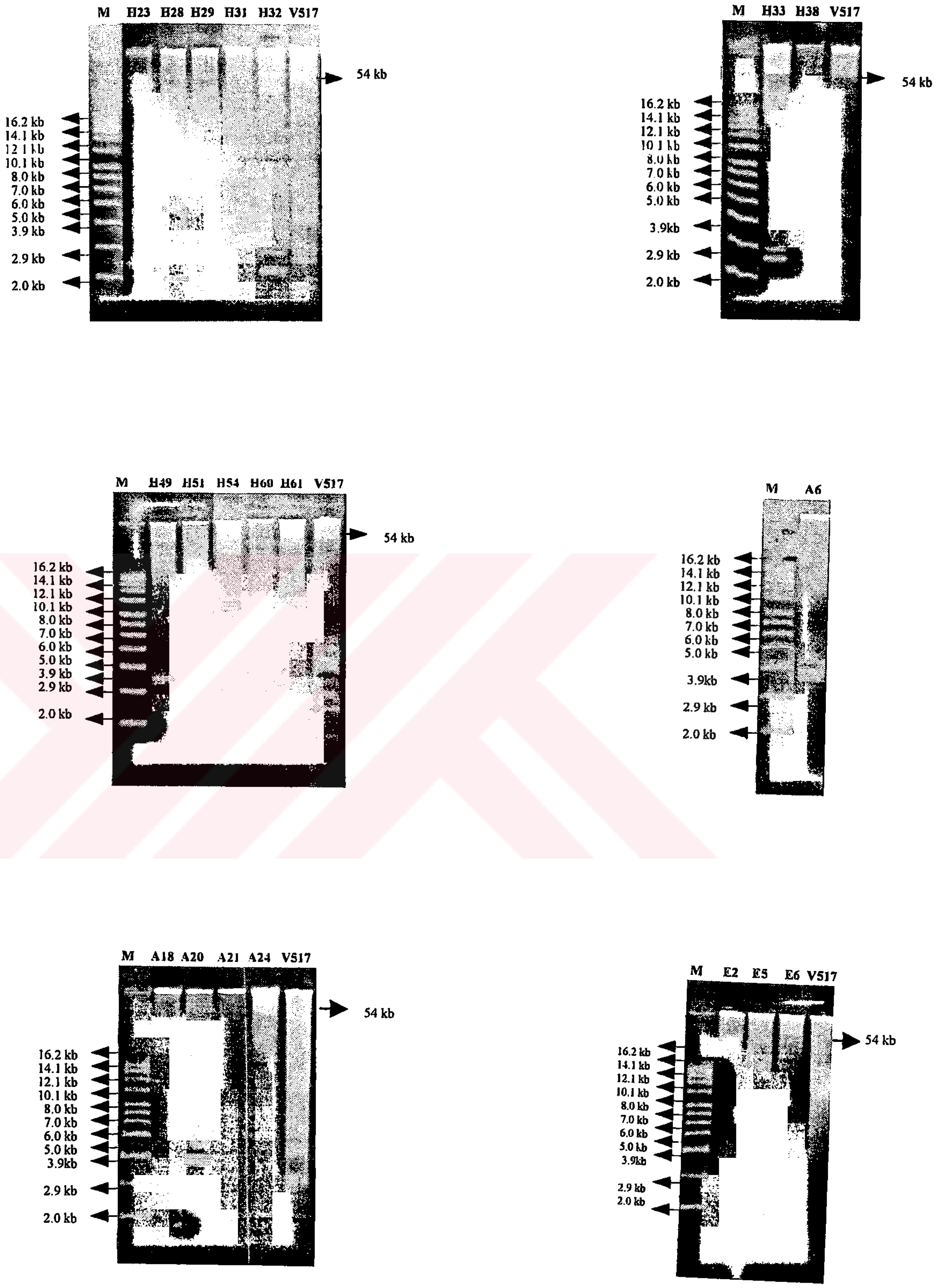


Figure 3.20. Plasmid profiles of the enterics other than *S. enteritidis*

3.4. Phage Typing

3.4.1. Phage Typing of *S. enteritidis* Human Isolates

Six phage types were identified within the 51 *S. enteritidis* human isolates (Table 3.11). The strains belonging to PT4 were predominating as they constituted 74.5% of all human isolates. The isolate H37 was untypable.

Table 3.11. PT profiles of *S. enteritidis* human isolates

Strain Designation	PT Profile	Number of Strains	% of Isolates
H13	1	1	1.96
H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H21, H25, H27, H30, H34, H35, H36, H39, H41, H42, H43, H44, H45, H46, H47, H48, H50, H52, H53, H55, H56, H57, H58, H59, H62, H63	4	38	74.5
H15, H16, H40,	6	3	5.88
H22, H24, H26	7	3	5.88
H14, H17, H18, H19	8	4	7.84
H20	13a	1	1.96
H37	UT*	1	1.96

UT* Untypable

3.4.2. Statistical Analysis of PhageTyping Data Obtained from *S. enteritidis* Human Isolates

When a dendogram was constructed by SAHN clustering, 4 main clusters were obtained from the phage typing data of *S. enteritidis* human isolates (Figure 3.21). The isolates H13, H37 and H20 were out of the clusters.

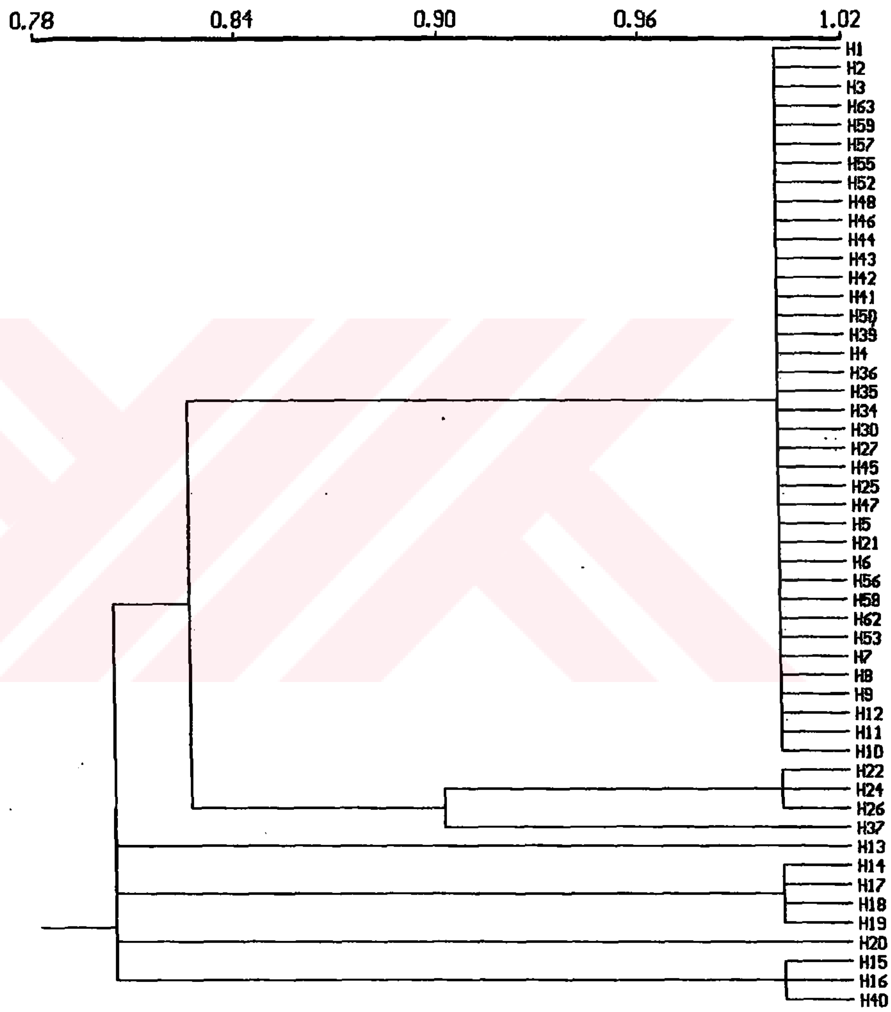


Figure 3.21. Dendogram constructed as based on phage typing of *S. enteritidis* human isolates

The PCA clustering pattern is shown in Figure 3.21 (Figure 3.22).

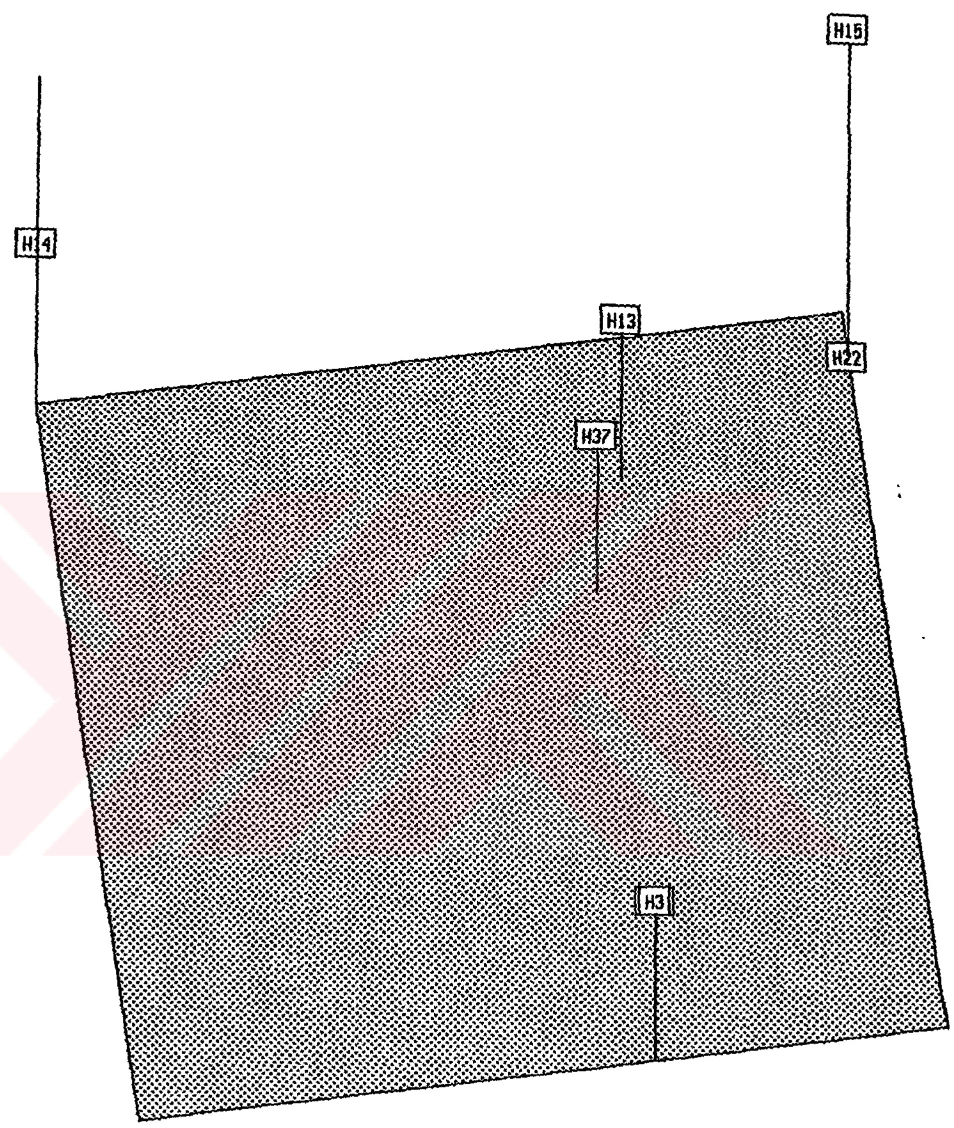


Figure 3.22. PCA of phage typing dataobtained from *S. enteritidis* human isolates in three dimensions

3.4.3. Phage Typing of *S. enteritidis* Chicken Isolates

Four phage types were identified within the 21 *S. enteritidis* chicken isolates (Table 3.12). The strains belonging to PT6 were predominating (71.42%). The isolates A22 and A23 were untypable.

Table 3.12. PT profiles of *S. enteritidis* chicken isolates

Strain Designation	PT Profile	Number of Strains	% of Isolates
A25, A26	1	2	9.52
A3	4	1	4.76
A1, A2, A4, A5, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A19	6	15	71.42
A17	35	1	4.76
A22, A23	UT*	2	9.52

UT* Untypable

3.4.4. Statistical Analysis of Phage Typing Data Obtained from *S. enteritidis* Chicken Isolates

Three main clusters were obtained from the dendogram of phage typing results of *S. enteritidis* chicken isolates (Figure 3.23). The isolates A3 and A17 were out of the clusters.

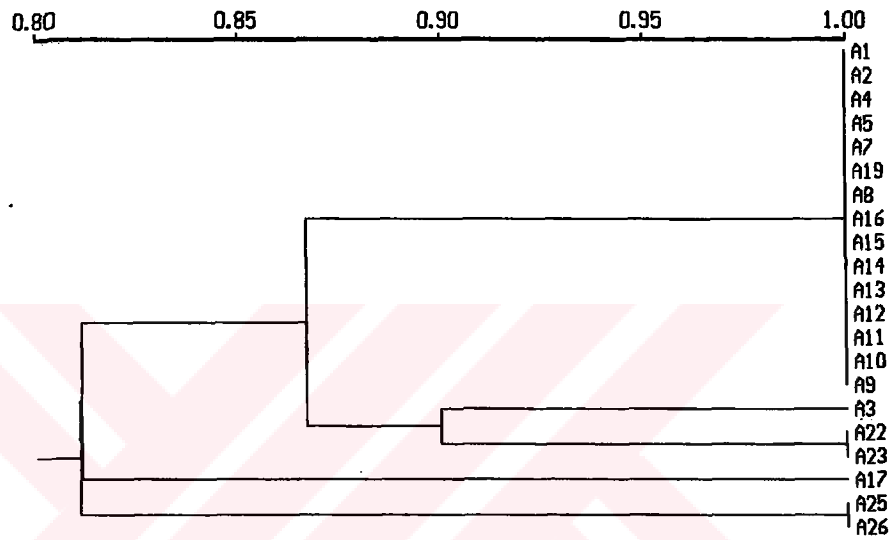


Figure 3.23 Dendogram constructed as based on phage typing results of *S. enteritidis* chicken isolates

PCA clustering also showed these three main clusters and the isolates A3 and A17 were out of the clusters (Figure 3.24)

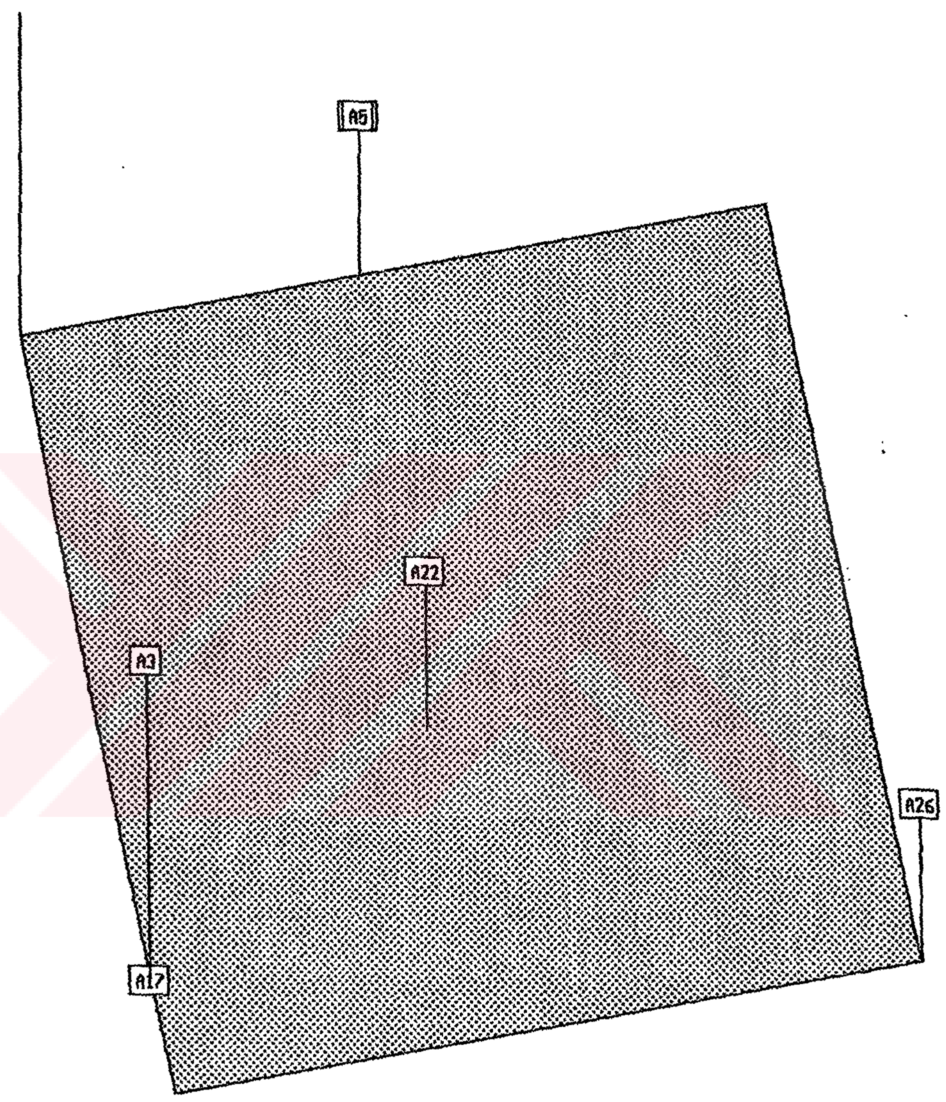


Figure 3.24. PCA clustering as based on phage typing data of *S. enteritidis* chicken isolates in three dimensions

3.4.5. Phage Typing of *S. enteritidis* Egg Isolates

Six phage types were identified for the 10 *S. enteritidis* egg isolates (Table 3.13). It was found that the strains belonging to PT4 were predominating (50%).

Table 3.13. PT profiles of *S. enteritidis* egg isolates

Strain Designation	PT Profile	Number of Strains	% of Isolates
E10	1	1	10
E1, E3, E4, E9, E13,	4	5	50
E12	7	1	10
E8	9c	1	10
E7	20a	1	10
E11	25	1	10

3.4.6. Statistical Analysis of Phage Typing Data Obtained from *S. enteritidis* Egg Isolates

The dendogram based on phage typing results of *S. enteritidis* egg isolates revealed one main cluster which included the isolates E1, E3, E4, E9 and E13 (Figure 3.25). The other isolates E7, E8, E10, E11 and E12 were out of the clusters.

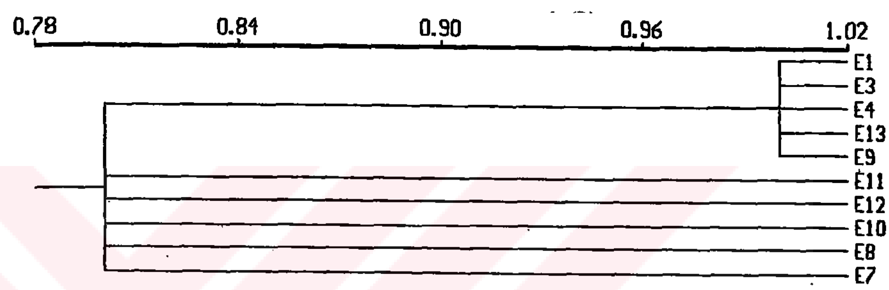


Figure 3.25. Dendogram constructed from the phage typing data obtained from *S. enteritidis* egg isolates

PCA was also applied to phage typing data obtained from *S. enteritidis* egg isolates (Figure 3.26).

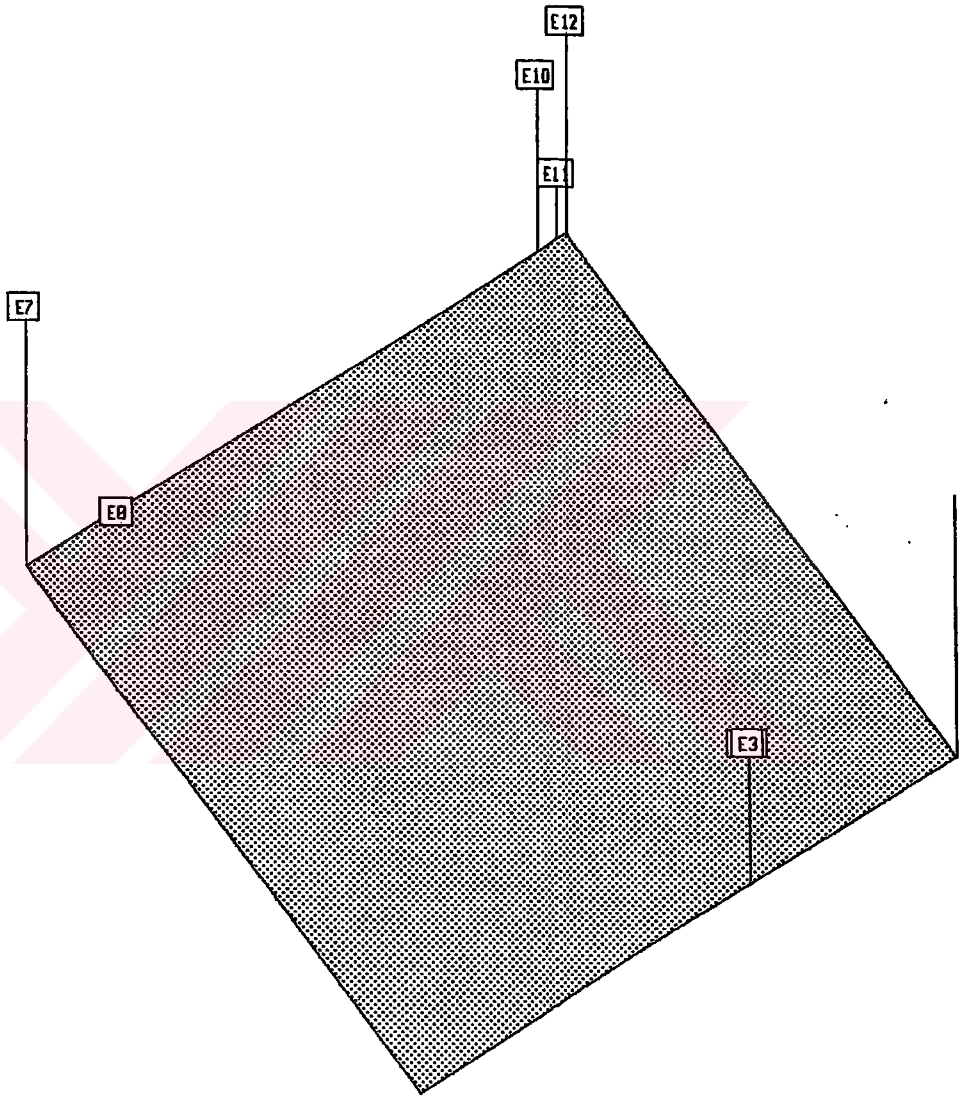


Figure 3.26. PCA applied to phage typing data from *S. enteritidis* egg isolates

3.4.7. A Summary of Phage Typing Results of *S. enteritidis* Strains

Table 3.14 represents a sum of the phage typing results of all isolates from three different sources examined. Ten phage types were identified within the 82 *S. enteritidis* strains as a total. Phage type 4 (PT4) was predominating (53.65%) in these strains which was followed by PT6 (21.95%). Egg isolates revealed six different types of PTs (PT1, 4, 7, 9, 20 and 25). It is to be noted that none of the egg isolates belonged to PT6. Human isolates also showed six different PTs (PT1, 4, 6, 7, 8, and 13a) while chicken isolates revealed four different types of PTs (PT1, 4, 6 and 35). Three of the 82 isolates were untypable.

Table 3.14. Summary of PT results from *S. enteritidis* strains

Strain Designation	PT Profile	Number of Strains	% of Isolates
H13, E10, A25, A26	1	4	4.87
H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H21, H25, H27, H30, H34, H35, H36, H39, H41, H42, H43, H44, H45, H46, H47, H48, H50, H52, H53, H55, H56, H57, H58, H59, H62, H63, E1, E3, E4, E9, E13, A3	4	44	53.65
H15, H16, H40, A1, A2, A4, A5, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A19	6	18	21.95
H22, H24, H26, E12	7	4	4.87
H14, H17, H18, H19	8	4	4.87
H20	13a	1	1.21
E8	9	1	1.21
E7	20	1	1.21
E11	25	1	1.21
A17	35	1	1.21
H37, A22, A23	UT*	3	3.65

UT* Untypable

3.4.8. Statistical Analysis of Phage Typing Data of *S. enteritidis* Strains

The dendogram of phage typing findings of all *S. enteritidis* isolates revealed 6 main clusters (Figure 3.27). The isolates A17, E7, H20, E8 and E11 were out of the clusters.

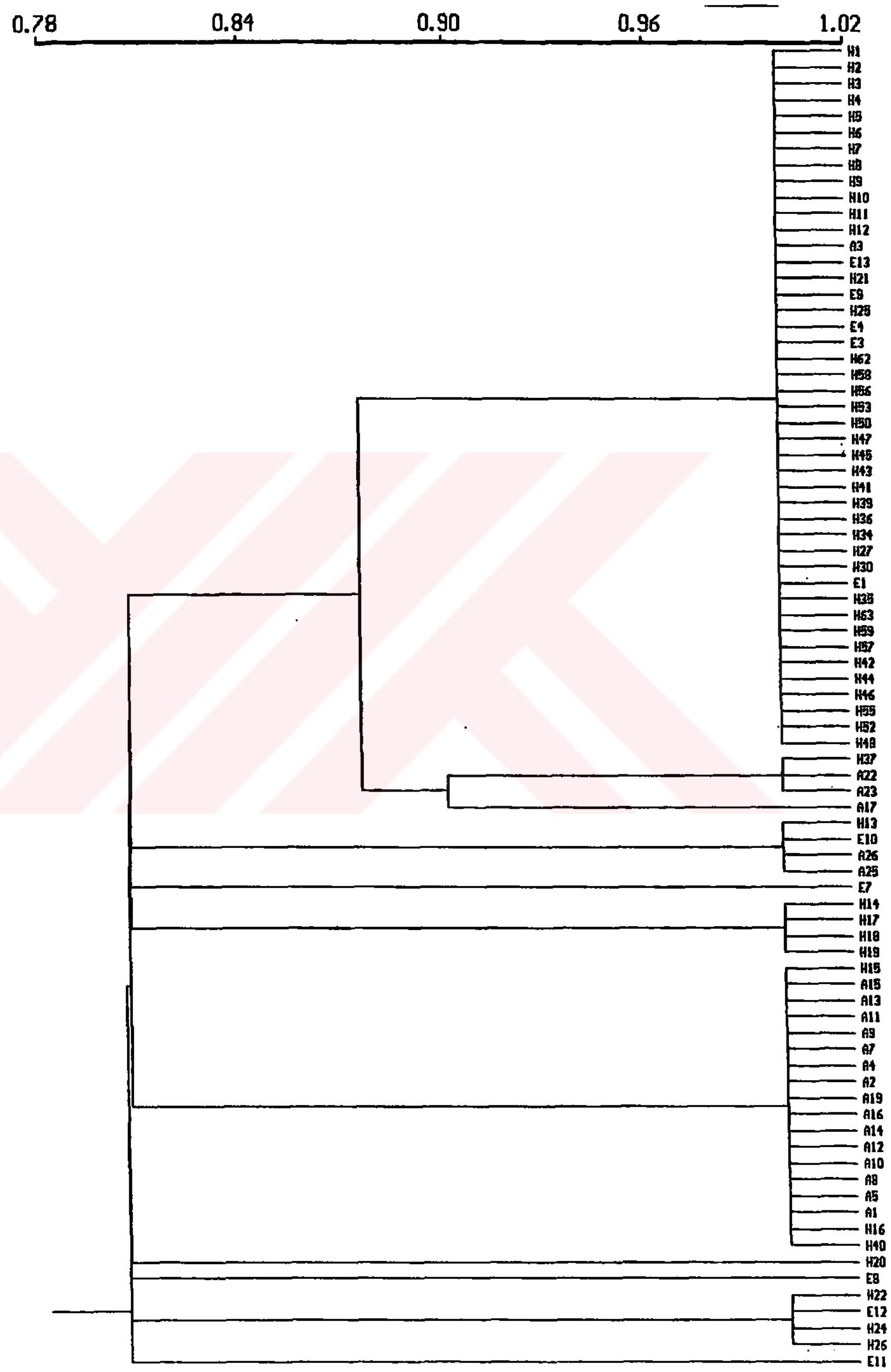


Figure 3.27. Dendogram constructed from the phage typing data obtained from *S. enteritidis* strains

PCA resulted in seven main clusters one of which was formed by untypable isolates A22, A23 and H37 (Figure 3.28).

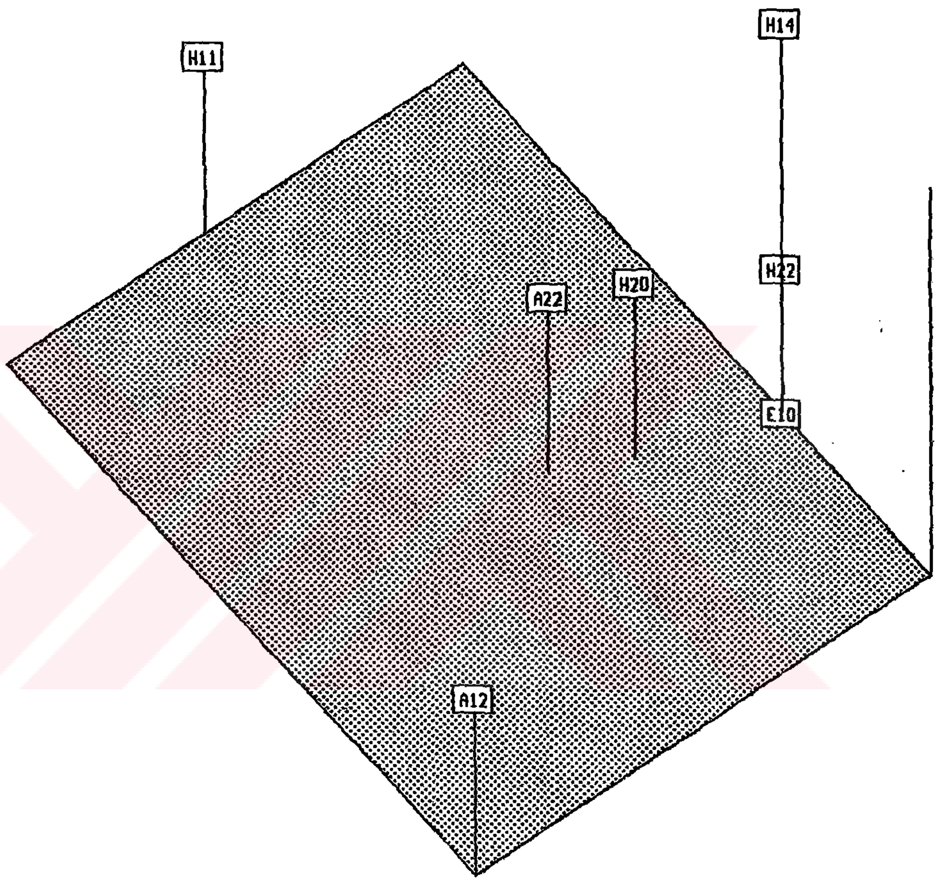


Figure 3.28. PCA of phage typing data obtained from of *S. enteritidis* strains.

3.4.9. Phage Typing Results Obtained from the Species of *Enterobacteriaceae* Other Than *S. enteritidis*

Phage typing is used to subdivide strains within a particular serotype and phage typing schemes have been developed for the most common salmonella serotypes, namely *S. enteritidis*, *S. typhimurium* and *S. virchow*, and also for other serotypes of clinical and epidemiological importance (Ward *et al.*, 1987). The phage types of enterics other than *S. enteritidis* which are also of clinical and epidemiological importance used in the study are given in Table 3.15.

Table 3.15. PT results of enterics used other than *S. enteritidis*

Strain Designation	Name of the Species	Source	PT or Serology
E2	*Salmonella kedougou*	Egg	I 13, 23: i: l, w
E5	*Salmonella*	Egg	I O=rough: g, m: -
E6	*Enterobacter*	Egg	UT*
A6	*Salmonella typhimurium*	Chicken	PT99
A18	*Salmonella ouakam*	Chicken	I 9, 46: z29
A20	*Salmonella typhimurium*	Chicken	PT164
A21	*Salmonella ouakam*	Chicken	I 9, 46: z29
A24	*Salmonella ouakam*	Chicken	I 9, 46: z29
H23	*Salmonella baildon*	Human	I 9, 46: a: e,n, x
H28	*Salmonella typhimurium*	Human	UT*
H29	*Salmonella typhimurium*	Human	PT104
H31	*Salmonella typhimurium*	Human	PT104
H32	*Salmonella havana*	Human	I 13, 23: f, g: -
H33	*Salmonella typhimurium*	Human	PT104c
H38	*Salmonella agona*	Human	PT1
H49	*Salmonella typhimurium*	Human	PT104
H51	*Citrobacter freundii*	Human	UT*
H54	*Enterobacter*	Human	UT*
H60	*Proteus*	Human	UT*
H61	*Citrobacter*	Human	UT*

UT* Untypable

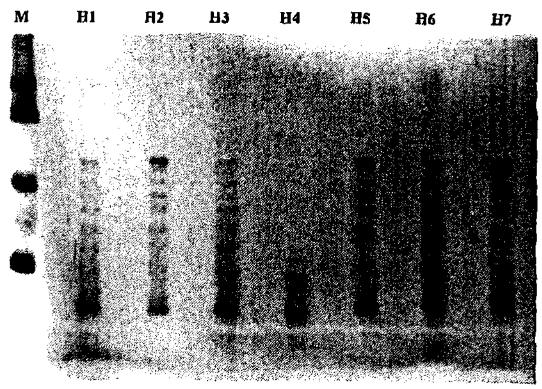
3.5. Lipopolysaccharide (LPS) Profiles

3.5.1. LPS Profiles of *S. enteritidis* Human Isolates

Bacteria which contain LPS that lacks the O-antigenic side chain often referred to as rough whereas bacteria which have this LPS component are referred to as smooth (Todar, 1997). SDS-PAGE analysis of LPS prepared from the 51 *S. enteritidis* human isolates showed that 41 (80.4%) of the *S. enteritidis* human isolates expressed long-chain LPS giving a characteristic 'ladder' pattern defined as 'smooth' (Figure 3.29). The remaining ten isolates (19.6%) did not produce LPS profiles with this ladder pattern, that is 'rough'. LPS patterns of *S. enteritidis* human isolates are documented in Table 3.16.

Table 3.16. LPS profiles of *S. enteritidis* human isolates

Strain Designation	LPS Profile	Number of Strains	% of Isolates
H8, H9, H10, H11, H12, H13,H14, H15, H16, H17, H18, H19, H20, H21, H25, H27, H30, H34, H35, H36, H37, H39, H40, H41, H42, H43, H44, H45, H46, H47, H48, H50, H52, H53, H55, H56, H57, H58, H59, H62, H63	Smooth	41	80.4
H1, H2, H3, H4, H5, H6, H7, H22, H24, H26	Rough	10	19.6



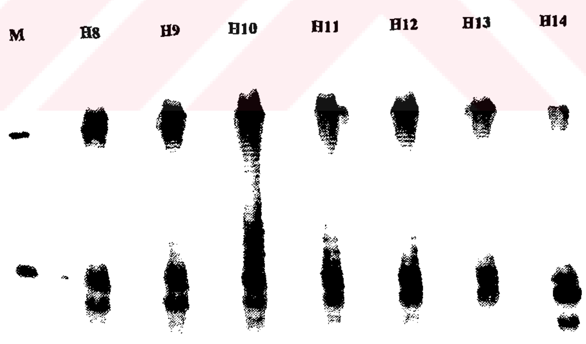


Figure 3.29. LPS profiles of *S. enteritidis* human isolates

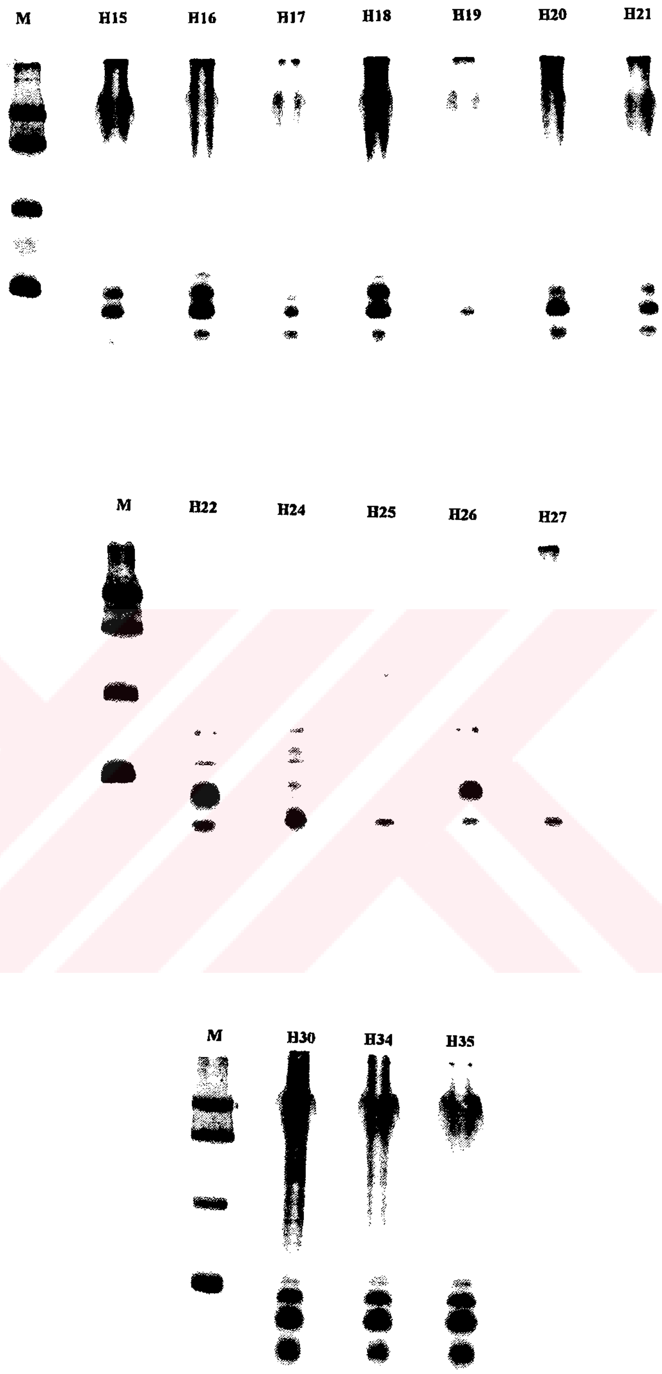
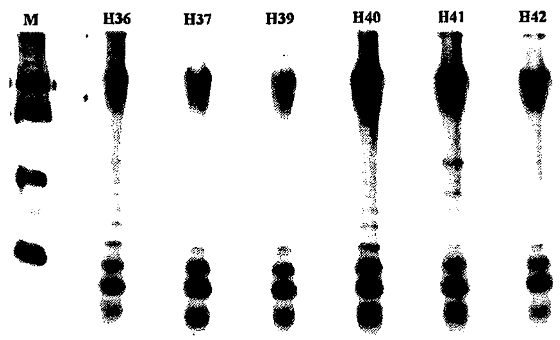


Figure 3.29. (Cont'd)



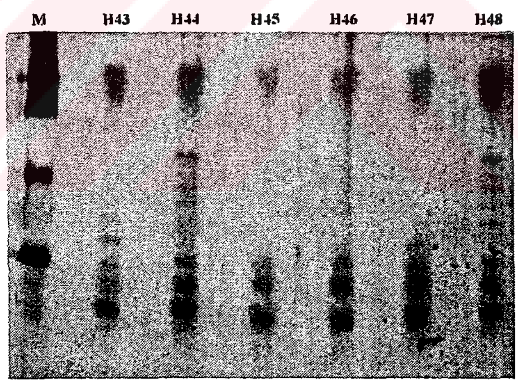
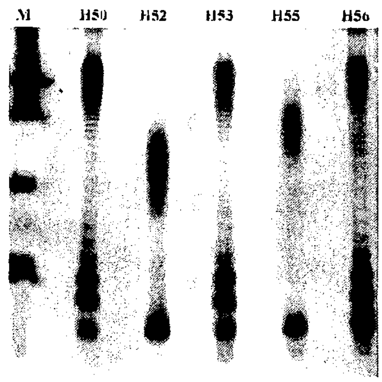


Figure 3.29. (Cont'd)



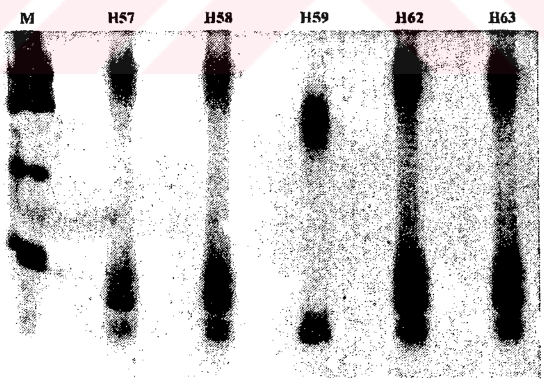


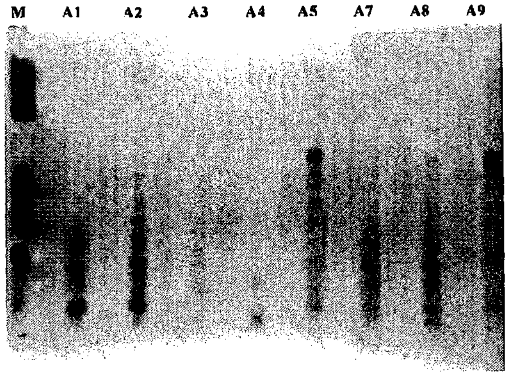
Figure 3.29. (Cont'd)

3.5.2. LPS Patterns of *S. enteritidis* Chicken Isolates

LPS patterns of *S. enteritidis* chicken isolates are given in Table 3.17. It was found that 5 (23.8%) out of the 21 chicken isolates expressed smooth type LPS (Figure 3.30). 76.2% of the chicken isolates were rough.

Table 3.17. LPS profiles of *S. enteritidis* chicken isolates

Strain Designation	LPS Profile	Number of Strains	% of Isolates
A10, A11, A13, A14, A15	Smooth	5	23.8
A1, A2, A3, A4, A5, A7, A8, A9, A12, A16, A17, A19, A22, A23, A25, A26	Rough	16	76.2



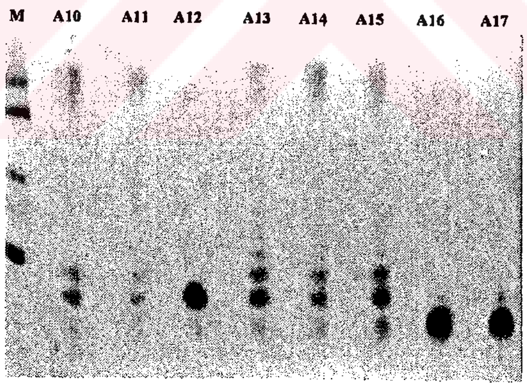


Figure 3.30. LPS profiles of *S. enteritidis* chicken isolates

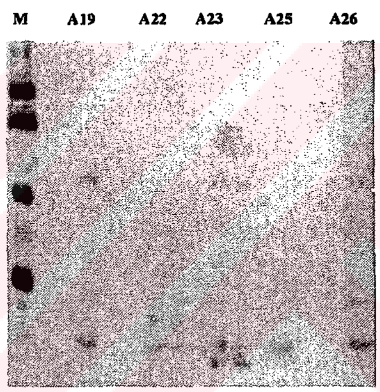


Figure 3.30. (Cont'd)

3.5.3. LPS Patterns of *S. enteritidis* Egg Isolates

LPS results of *S. enteritidis* egg isolates are given in Table 3.18. 4 (40%) out of the 10 egg isolates expressed smooth type LPS (Figure 3.31). The isolates E8, E9, E10, E11, E12 and E13 were rough.

Table 3.18. LPS profiles of *S. enteritidis* egg isolates

Strain Designation	LPS Profile	Number of Strains	% of Isolates
E1, E3, E4, E7	Smooth	4	40
E8, E9, E10, E11, E12, E13	Rough	6	60

Figure 3.31. LPS profiles of *S. enteritidis* egg isolates

3.5.4. A Summary of LPS of *S. enteritidis* Strains from Different Sources

A sum of LPS profiles of *S. enteritidis* isolates from three different sources are given in Table 3.19. 50 (60.97%) of the 82 *S. enteritidis* isolates expressed long-chain LPS giving a charateristic 'ladder' pattern (smooth). A higher percentage of the chicken and egg isolates showed rough type LPS (60% and 76.2%, respectively) while most of the human isolates (80.4%) expressed smooth type LPS.

Table 3.19. LPS profiles of *S. enteritidis* isolates

Sources of Isolates	Numbers of Isolates	Number of Isolates Having Smooth LPS Profile (%)	Number of Isolates Having Rough LPS Profile (%)
Egg	10	4(%40)	6(%60)
Chicken	21	5(%23.8)	16(%76.2)
Human	51	41(%80.4)	10(%19.6)

3.5.5. LPS Patterns *Enterobacteriaceae* Species Other Than *S. enteritidis*

The LPS profiles of the enterics other than *S. enteritidis* used in the study are shown in Figure 3.32 and summarized in Table 3.20. 14 of the 20 isolates expressed smooth type LPS.

Table 3.20. LPS profiles of enteric species other than *S. enteritidis*

Strain Designation	Name of the Species	Source	LPS Profile
E2	*Salmonella kedougou*	Egg	Smooth
E5	*Salmonella*	Egg	Rough
E6	*Enterobacter*	Egg	Smooth
A6	*Salmonella typhimurium*	Chicken	Rough
A18	*Salmonella ouakam*	Chicken	Smooth
A20	*Salmonella typhimurium*	Chicken	Rough
A21	*Salmonella ouakam*	Chicken	Rough
A24	*Salmonella ouakam*	Chicken	Rough
H23	*Salmonella baildon*	Human	Smooth
H28	*Salmonella typhimurium*	Human	Smooth
H29	*Salmonella typhimurium*	Human	Smooth
H31	*Salmonella typhimurium*	Human	Smooth
H32	*Salmonella havana*	Human	Smooth
H33	*Salmonella typhimurium*	Human	Smooth
H38	*Salmonella agona*	Human	Smooth
H49	*Salmonella typhimurium*	Human	Smooth
H51	*Citrobacter freundii*	Human	Smooth
H54	*Enterobacter*	Human	Smooth
H60	*Proteus*	Human	Rough
H61	*Citrobacter*	Human	Smooth

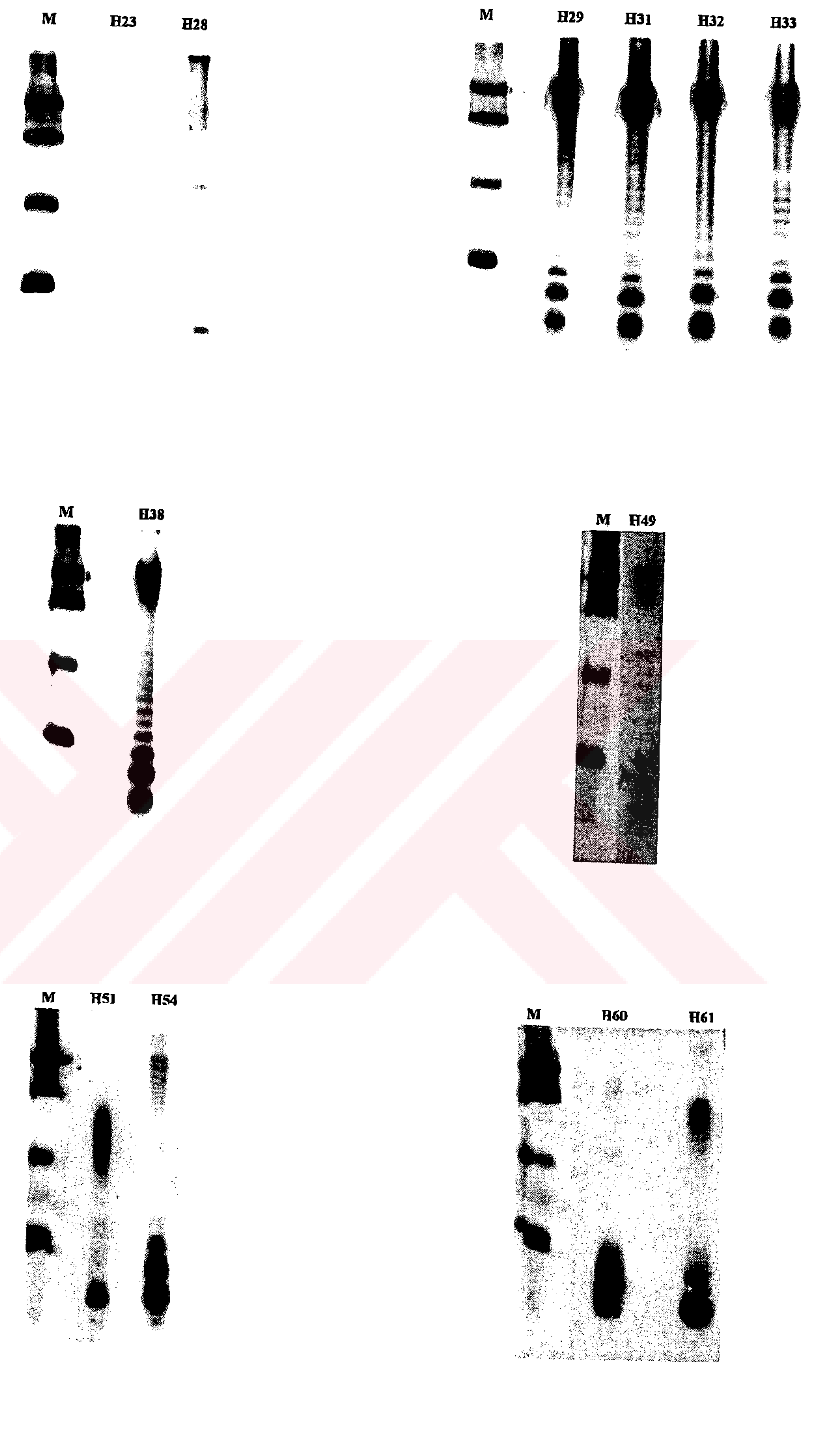


Figure 3.32. LPS profiles of other enterics used in the study

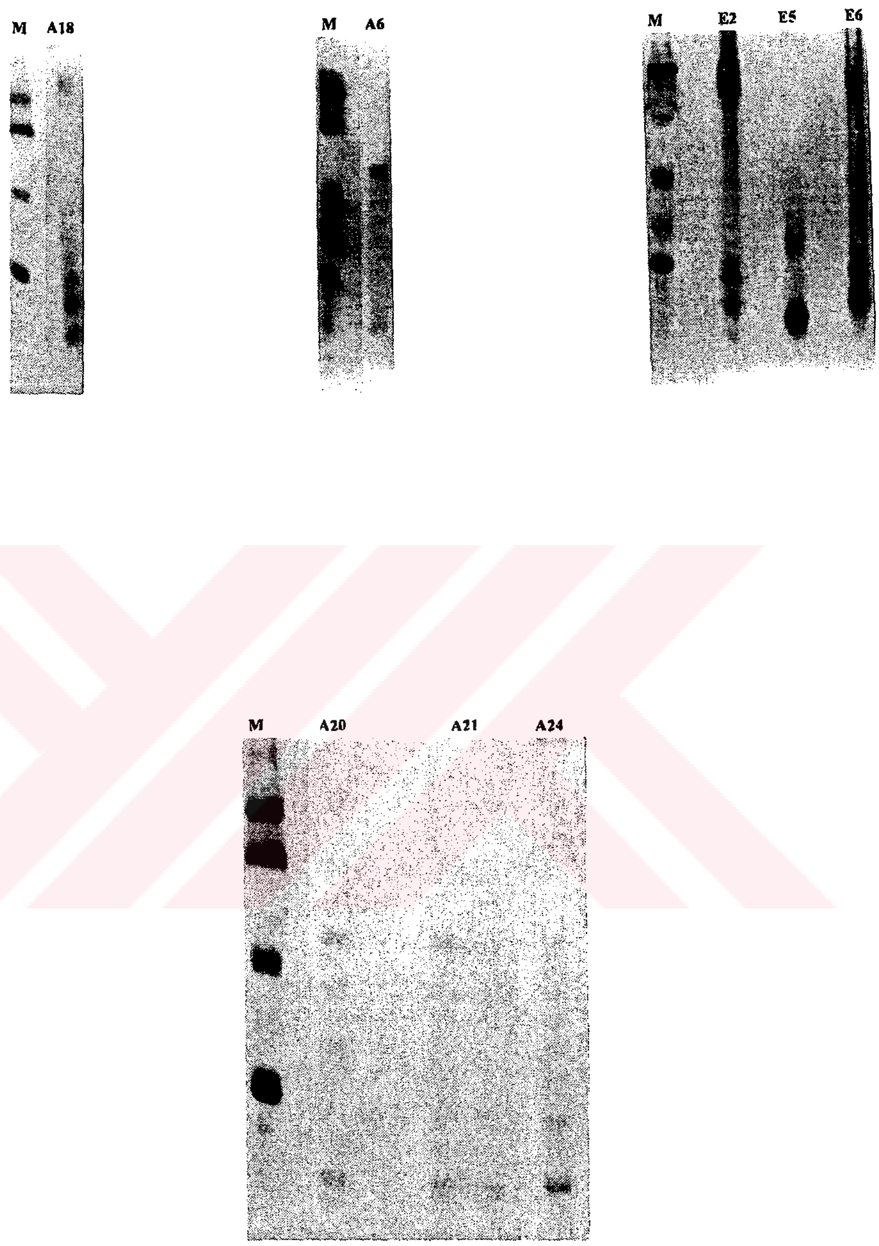


Figure 3.32. (Cont'd)

3.6. Outer Membrane Protein (OMP) Isolation Results

The nomenclature of the OMPs in OMP-typing schemes has not been standardized. Therefore such schemes are generally developed by individual research groups. We also developed an OMP-typing scheme which is based on inter-strain variation of the major outer membrane proteins (MOMP). Four distinct OMPs were found to be harbored by the *S. enteritidis* isolates examined. Based on this, we defined four putative OMP types; OMP-typeI, OMP-typeII, OMP-typeIII and OMP-typeIV, respectively. Isolates of these types were expected to possess 34.61 kDa, 30.65 kDa, 27.15 kDa and 25.56 kDa proteins, respectively. These OMP-types that we defined are represented schematically in Figure 3.33.

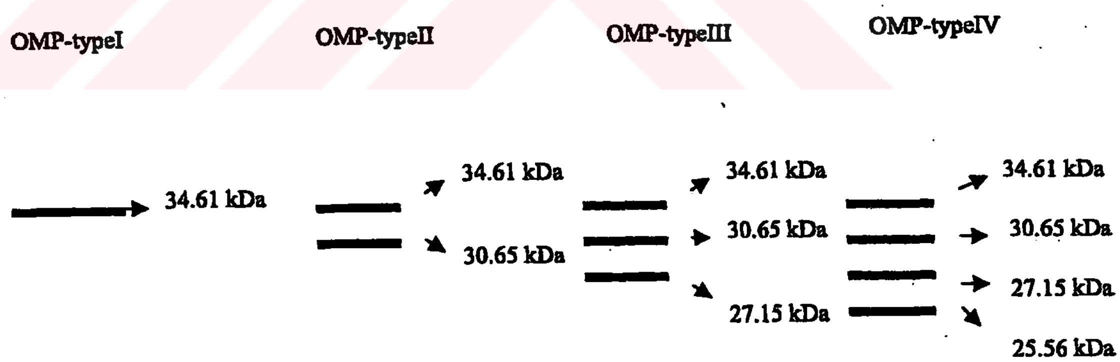


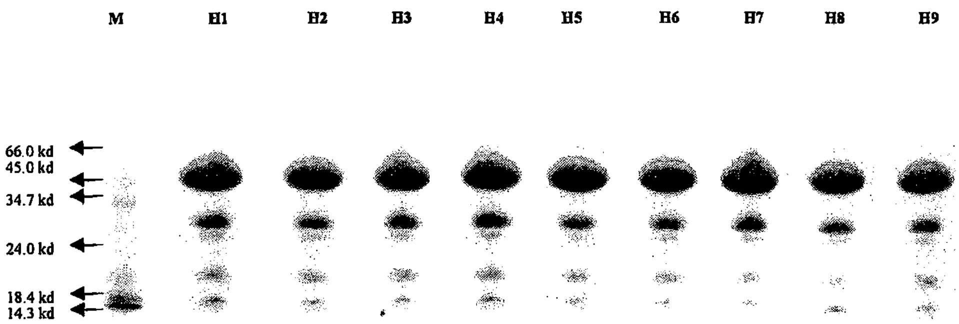
Figure 3.33. Schematic representation of OMP-types of *S. enteritidis*

3.6.1. OMP Types of *S. enteritidis* Human Isolates

The OMP types of *S. enteritidis* human isolates are shown in Figure 3.34 and summarized in Table 3.21. Of the 51 *S. enteritidis* human isolates examined, 35 (68.62%) were of OMP-typeII and 16 (31.37%) were of OMP-typeIII. None of the *S. enteritidis* human isolates belonged to OMP-typeI or OMP-typeIV.

Table 3.21. OMP profiles of *S. enteritidis* human isolates

OMP Type	Strain Designation	Number of Strains	% of Isolates
Type I		0	0
Type II	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H15, H17, H30, H34, H36, H37, H39, H40, H41, H42, H43, H44, H45, H50, H52, H55, H56, H57, H58, H59, H62, H63	35	68.62
Type III	H14, H16, H18, H19, H20, H21, H22, H24, H25, H26, H27, H35, H46, H47, H48, H53	16	31.37
Type IV		0	0



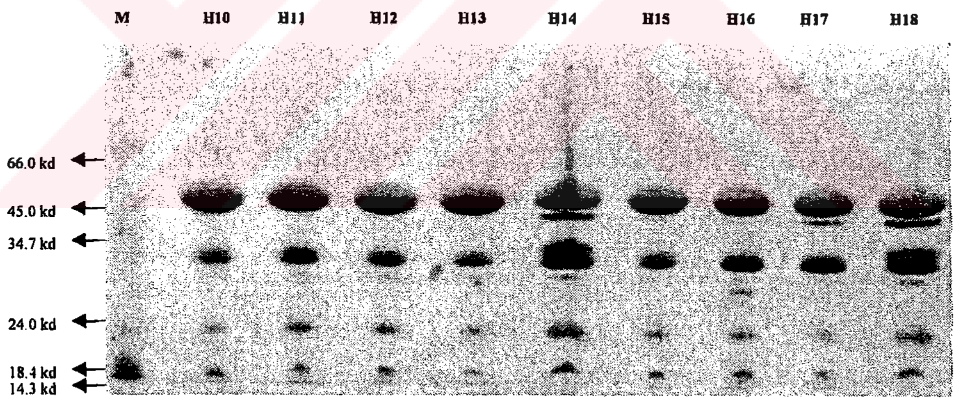
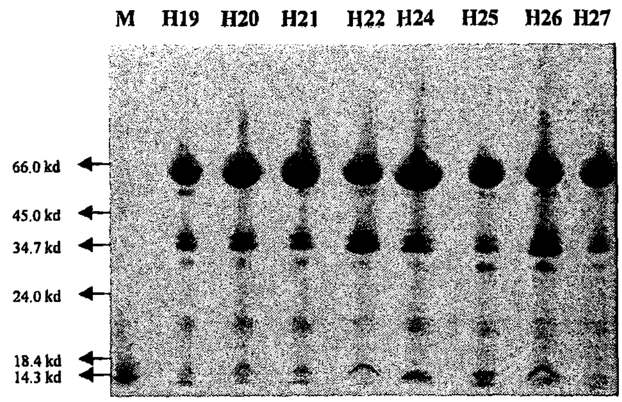


Figure 3.34. OMP profiles of *S. enteritidis* human isolates



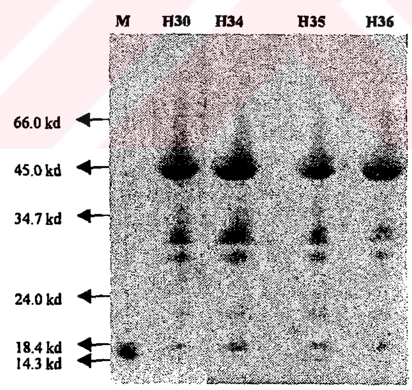
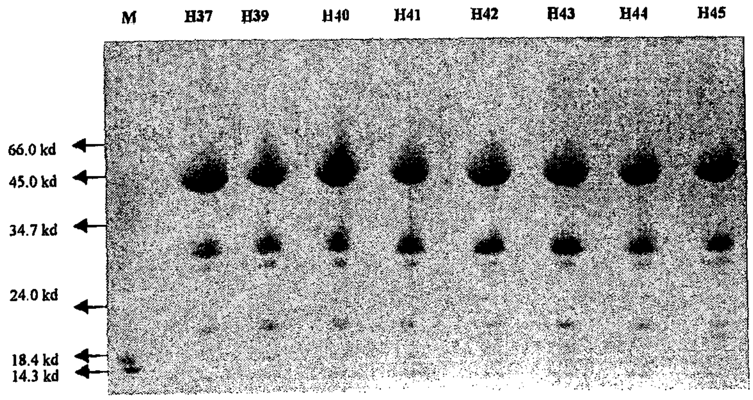


Figure 3.34. (Cont'd)



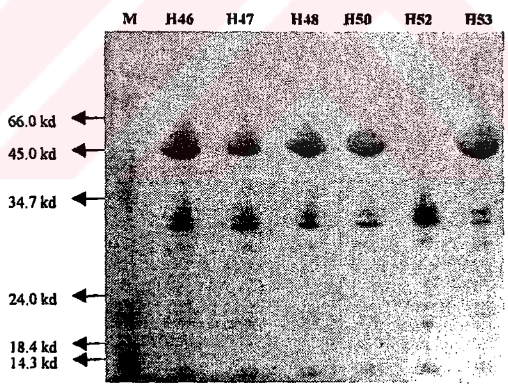


Figure 3.34. (Cont'd)

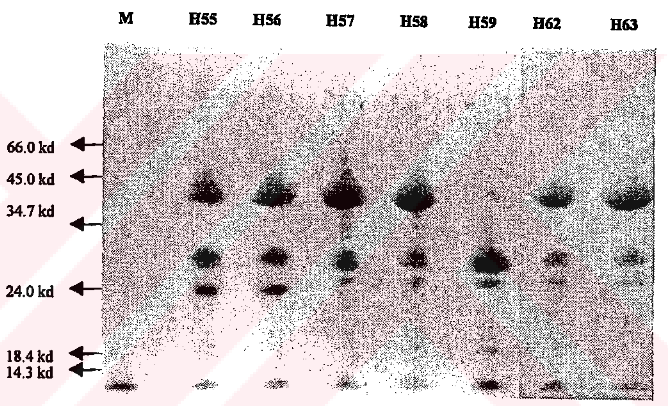


Figure 3.34. (Cont'd)

3.6.2. OMP Profiles of *S. enteritidis* Chicken Isolates

The OMP-types of *S. enteritidis* chicken isolates are shown in Figure 3.35 and summarized in Table 3.22. Of the 21 *S. enteritidis* chicken isolates examined, 11 (52.38%) were of OMP-typeIII, 8 (38.09%) were of OMP-typeII and 2 (9.52%) were of OMP-typeIV, respectively. None of the chicken isolates showed OMP-typeI.

Table 3.22. OMP profiles of *S. enteritidis* chicken isolates

OMP Type	Strain Designation	Number of Strains	% of Isolates
Type I		0	0
Type II	A1, A2, A4, A5, A7, A8, A25, A26	8	38.09
Type III	A3, A10, A11, A12, A13, A14, A15, A16, A17, A19, A23	11	52.38
Type IV	A9, A22	2	9.52

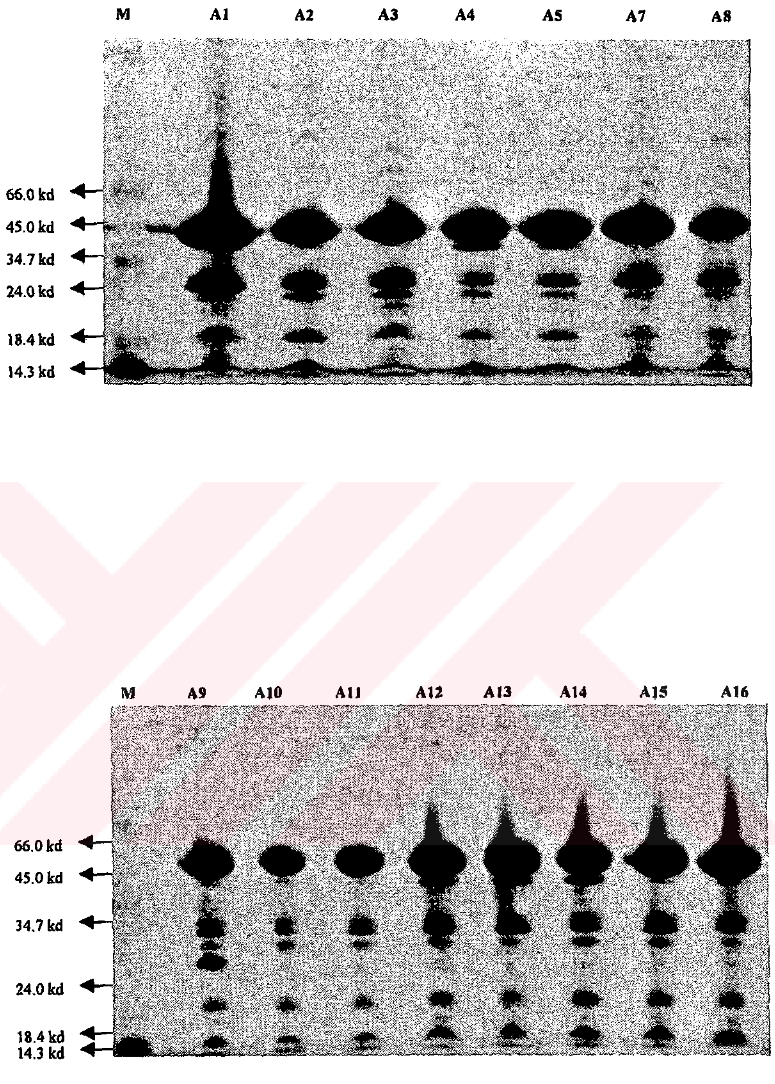
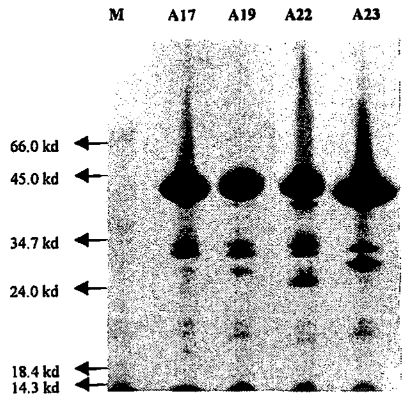


Figure 3.35 OMP profiles of *S. enteritidis* chicken isolates



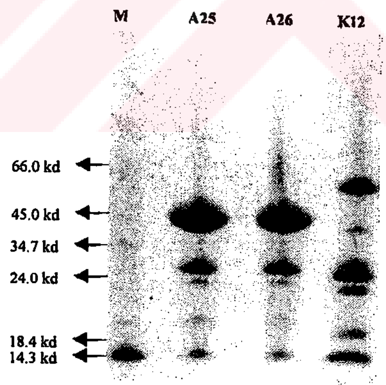


Figure 3.35. (Cont'd)

3.6.3. OMP Patterns of *S. enteritidis* Egg Isolates

The OMP-types of *S. enteritidis* egg isolates are documented in Figure 3.36 and summarized in Table 3.23. All of the *S. enteritidis* isolates were found to be OMP-typeII.

Table 3.23. OMP profiles of *S. enteritidis* egg isolates

OMP Type	Strain Designation	Number of Strains	% of Isolates
Type I		0	0
Type II	E1, E3, E4, E7, E8, E9, E10, E11, E12, E13	10	100
Type III		0	0
Type IV		0	0

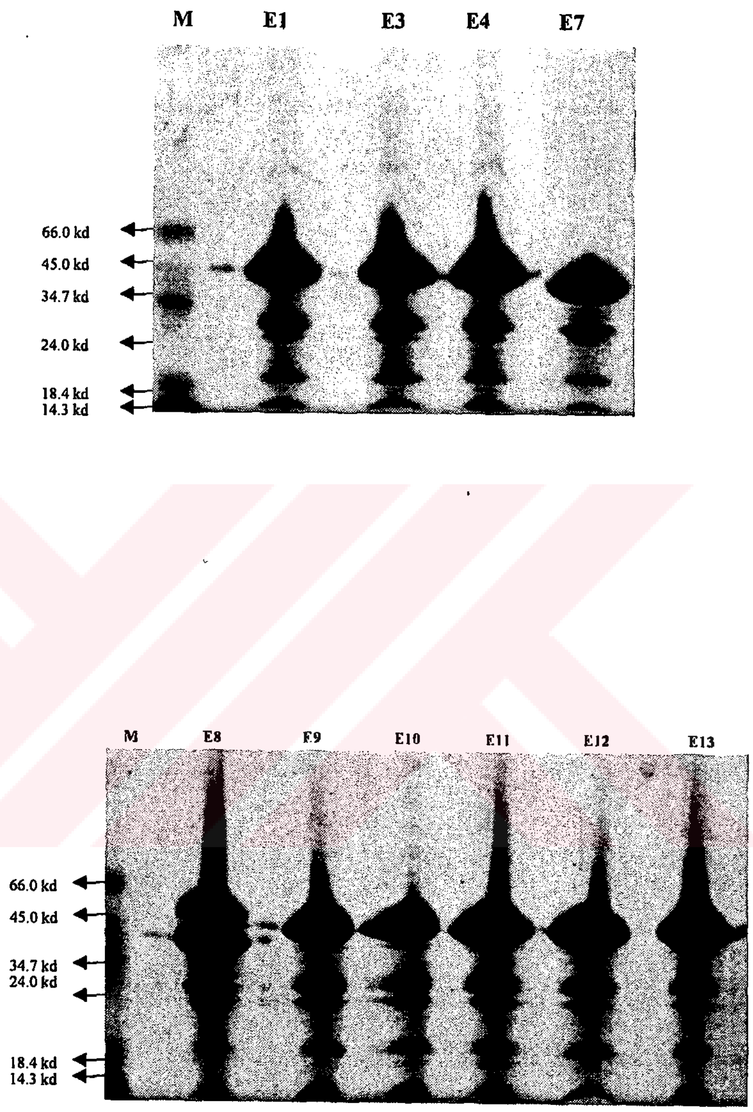


Figure 3.36. OMP profiles of *S. enteritidis* egg isolates

3.6.4. Summary of OMP Patterns of *S. enteritidis* Strains from Different Sources

A sum of OMP profiles of *S. enteritidis* isolates are given in Table 3.24. 53 (64.63%) of the 82 *S. enteritidis* isolates were OMP-typeII, 27 (32.92%) isolates were OMP-typeIII and 2 (2.43)% isolates were OMP-typeIV. All of the egg isolates were found to be OMP-typeII.

Table 3.24. Summary of OMP profiles of *S. enteritidis* strains

Source of Isolate	Number of Isolate	OMP-type (%)			
		I	II	III	IV
Human	51		35(68.62%)	16(31.37%)	
Chicken	21		8(38.09%)	11(52.38%)	2(9.52%)
Egg	10		10(100%)		
Total	82		53(64.63%)	27(32.92%)	2(2.43%)

3.6.5. OMP Patterns of Other Enteric Bacteria

The OMP profiles of the other enterics are shown in Figure 3.37 and summarized in Table 3.25. Of the 20 Enterics examined, 12 were of OMP-typeII, 7 were of OMP-typeIII, and one was of OMP-typeIV, respectively.

Table 3.25. OMP profiles of the other enterics used in the study

Strain Designation	Name of the Species	Source	OMP Type
E2	*Salmonella kedougou*	Egg	II
E5	*Salmonella*	Egg	II
E6	*Enterobacter*	Egg	III
A6	*Salmonella typhimurium*	Chicken	II
A18	*Salmonella ouakam*	Chicken	III
A20	*Salmonella typhimurium*	Chicken	II
A21	*Salmonella ouakam*	Chicken	III
A24	*Salmonella ouakam*	Chicken	III
H23	*Salmonella baildon*	Human	III
H28	*Salmonella typhimurium*	Human	II
H29	*Salmonella typhimurium*	Human	II
H31	*Salmonella typhimurium*	Human	II
H32	*Salmonella havana*	Human	II
H33	*Salmonella typhimurium*	Human	II
H38	*Salmonella agona*	Human	II
H49	*Salmonella typhimurium*	Human	II
H51	*Citrobacter freundii*	Human	II
H54	*Enterobacter*	Human	III
H60	*Proteus*	Human	IV
H61	*Citrobacter*	Human	III

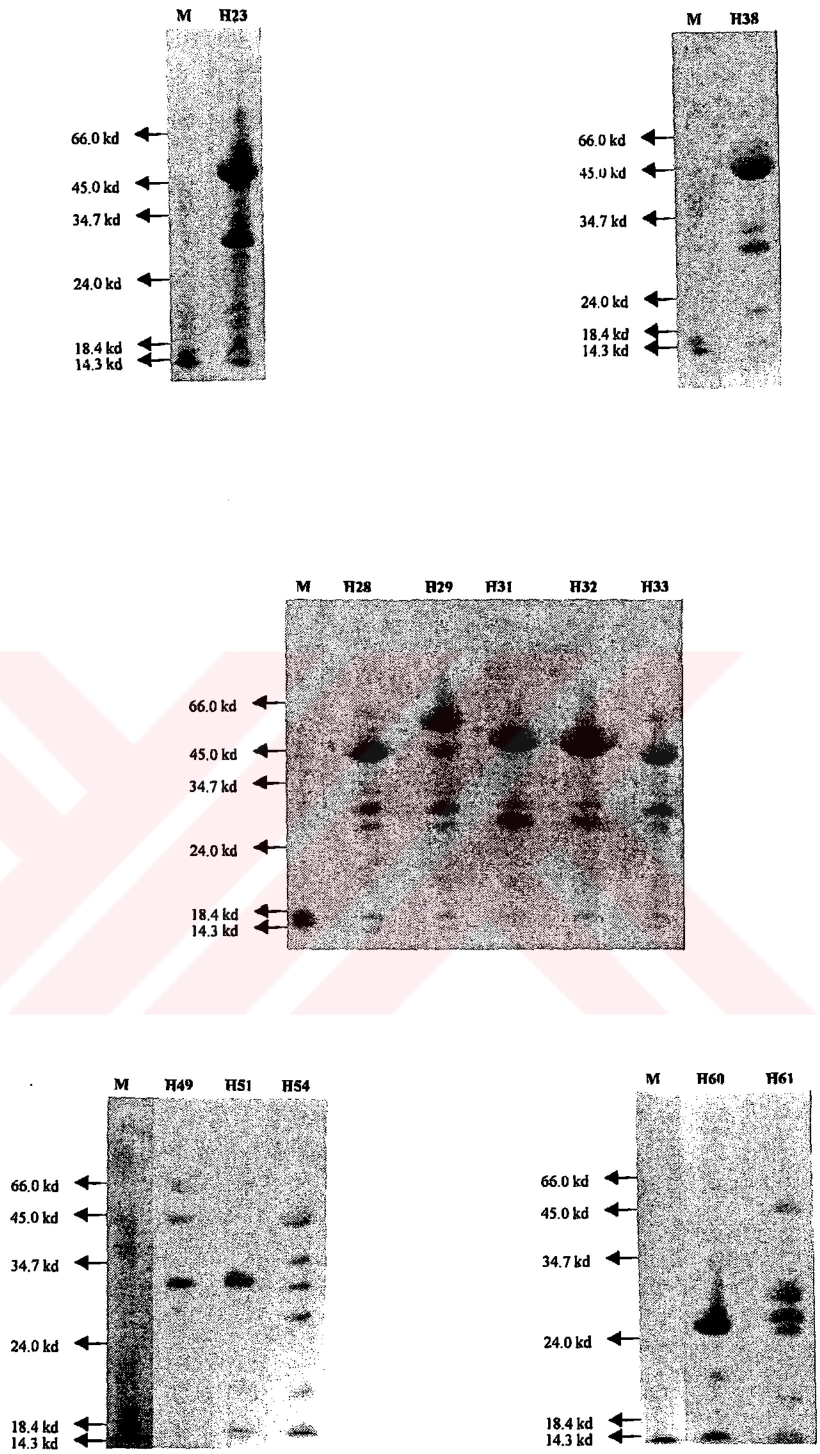


Figure 3.37. OMP profiles of the other enterics used in the study

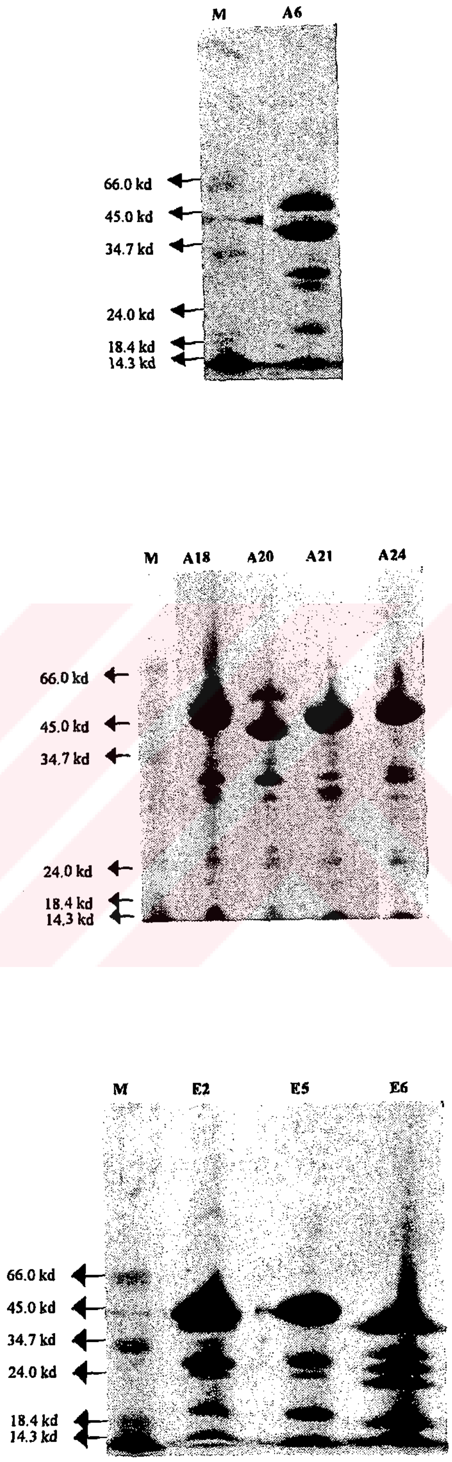


Figure 3.37. (Cont'd)

3.7. Comparison of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Isolates

3.7.1. Comparison of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Human Isolates

Table 3.26 represents a sum of the comparision of antibiotic resistance, plasmid, LPS, PT and OMP profiles of *S. enteritidis* human isolates. 31 different profiles were identified within the 51 such isolates for all the parameters tested. Human isolates with OMP-typeII outer membrane proteins showed four different phage types; PT4, PT1, PT6 and PT8. On the other hand, all the PT7 human isolates showed OMP-typeIII outer membrane protein and rough LPS profile. All the multiresistant human isolates expressed smooth type LPS profile. There was not a direct relation between plasmid content and antibiotic resistance patterns. The isolate H52, for instance, was multiresistant without harboring any plasmids.

Table 3.26. Comparison of antibiotic resistance, plasmid, LPS, OMP and PT profiles of *S. enteritidis* human isolates

Strain Designation	Source	Number of Strains	Plasmid Profile in kb	Antibiotic Resistance Profile	LPS Profile	OMP Type	PT
H1	Human	1	2.4; 1.9	TE	Rough	II	4
H2, H4, H6, H7	Human	4	54; 2.4; 1.9		Rough	II	4
H3	Human	1	2.4; 1.9		Rough	II	4
H22	Human	1	54; 23		Rough	III	7
H5	Human	1	54; 2.4; 1.9	PRL	Rough	II	4
H8	Human	1	54; 4.4; 2.4; 1.4	CKF	Smooth	II	4
H9, H11	Human	2	54; 2.4; 1.9	TE	Smooth	II	4
H21	Human	1	54; 2.4; 1.10	TE	Smooth	III	4
H53	Human	1	54; 27.5; 9.5	TE	Smooth	III	4
H10, H34, H41, H50	Human	4	54	TE; SXT	Smooth	II	4
H12, H36, H39, H42, H43, H45, H58, H63	Human	8	54	TE	Smooth	II	4
H20	Human	1	54	TE	Smooth	III	13a
H48	Human	1	54	TE	Smooth	III	4
H13	Human	1	54	AMC; AMP; CAR; CEC; CKF; CEP; PRL; SAM; TE	Smooth	II	1
H14	Human	1	54	AMC; AMP; CAR; CKF; CEP; PRL; SP; SAM; TE	Smooth	III	8
H15	Human	1	54; 4.4; 2.6; 1.9; 1.2	TE	Smooth	II	6
H59	Human	1	54; 4.1; 2.1; 1.6; 0.9	TE	Smooth	II	4
H16	Human	1	54; 33.8	AMC; AMP; CAR; CKF; CXM; PRL; SAM; TE	Smooth	III	6
H17	Human	1	54		Smooth	II	8
H30, H44	Human	2	54		Smooth	II	4
H18, H19	Human	2	54		Smooth	III	8
H25, H27, H35, H46, H47	Human	5	54		Smooth	III	4
H24	Human	1	54	CKF; TE	Rough	III	7
H26	Human	1	54	TE	Rough	III	7
H37	Human	1	54; 4.8; 3.9		Smooth	II	UT
H40	Human	1	54; 3.2		Smooth	II	6
H52	Human	1		AMC; CEC; CKF; CXM; C; CFM; TE; SXT	Smooth	II	4
H55	Human	1	54; 27.5; 13; 9.5; 5; 4.1; 2.1; 1.6; 1.3; 0.9	AMP; CKF	Smooth	II	4
H56	Human	1	54; 3.2	CKF; CXM; TE	Smooth	II	4
H57	Human	1	54	CKF; CXM; TE; SXT	Smooth	II	4
H62	Human	1	54	AMP; AZT; CEC; CKF; CXM; PRL; TE	Smooth	II	4

UT* Untypable

3.7.2. Statistical Analysis of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Human Isolates

When a dendogram was constructed to cover all the parameters tested, *S. enteritidis* human isolates gave rise to three main clusters (Figure 3.38) The isolates H52, H8, H59, H15, H16, H62, H13, H14 and H55 were out of the clusters.

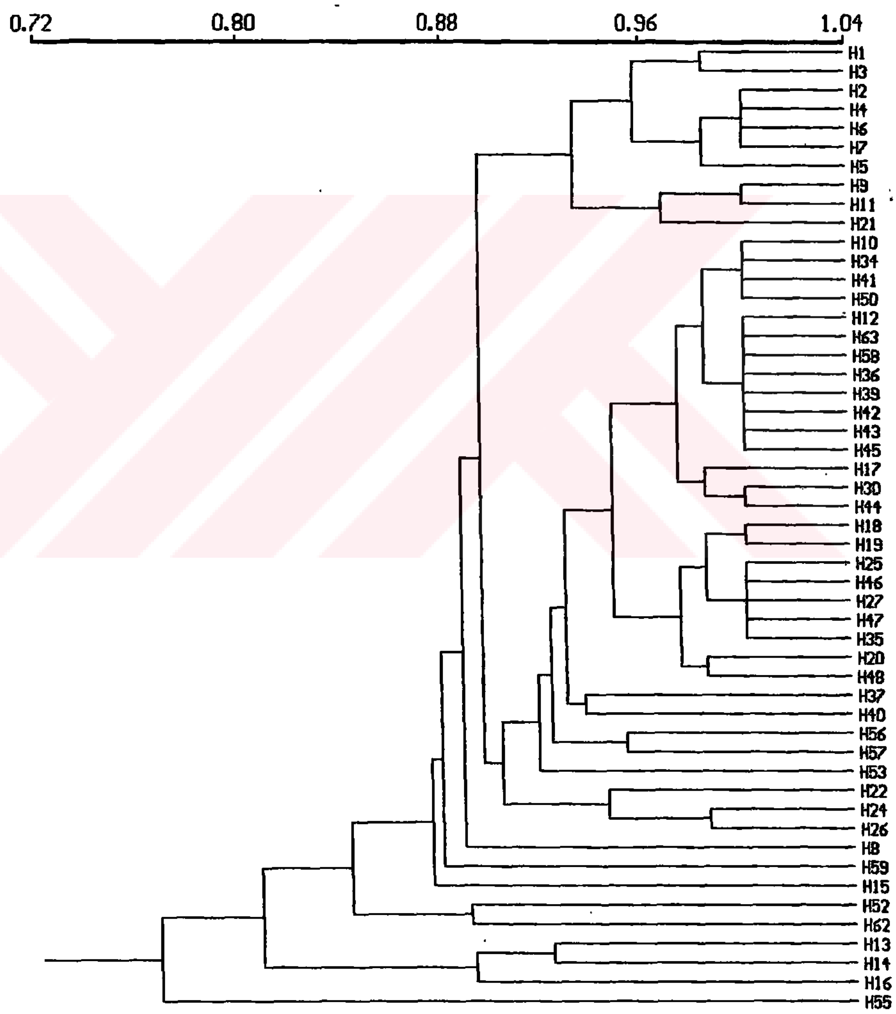


Figure 3.38. Dendogram constructed to cover all the parameters tested for *S. enteritidis* human isolates

PCA was also applied to the data of all the parameters tested *S. enteritidis* human isolates (Figure 3.39).

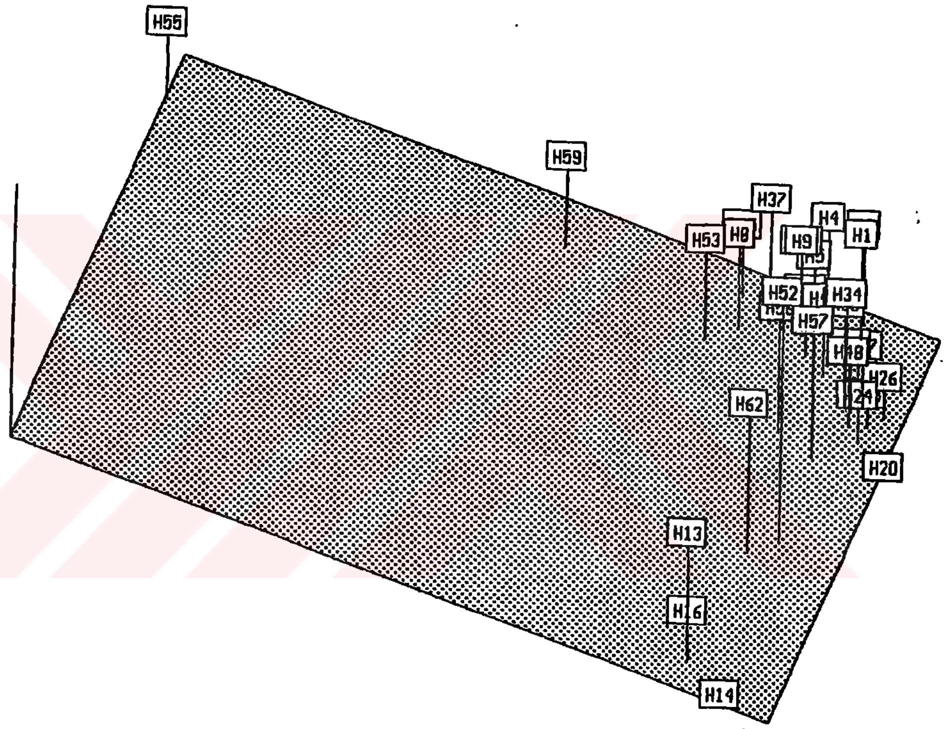


Figure 3.39. PCA data of all the parameters tested *S. enteritidis* human isolates in three dimensions

3.7.3. Comparison of of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Chicken Isolates

A sum of the comparison of antibiotic resistance, plasmid, LPS, PT and OMP profiles of *S. enteritidis* chicken isolates are given in Table 3.27. 20 different profiles were identified within the 21 *S. enteritidis* chicken isolates all the parameters tested. PT6 chicken isolates showed three different outer membrane protein profiles, OMP-typeII, OMP-typeIII and OMP-typeIV. All the phage types except PT6 expressed rough LPS profile. A direct relationship between plasmid content and antibiotic resistance could not be established. Although the isolates A9, A12 and A13 harbored high molecular weight plasmids they did not have resistance to any antibiotics.

Table 3.27. Comparison of antibiotic resistance, plasmid, LPS, OMP and PT profiles of *S. enteritidis* chicken isolates

Strain Designation	Source	Number of Strains	Plasmid Profile in kb	Antibiotic Resistance Profile	LPS Profile	OMP Type	PT
A1	Chicken	1	54; 22.9	AMC; AMP; CAR; CKF; CXM; PRL; SAM; TE	Rough	II	6
A2	Chicken	1	54; 22.9	AMP; CAR; CKF; CXM; PRL; TE	Rough	II	6
A3	Chicken	1	54	TE	Rough	III	4
A4	Chicken	1	54	AMP; CAR; TE	Rough	II	6
A19	Chicken	1	31.9	AMP; CKF; PRL	Rough	III	6
A5	Chicken	1	54; 28.7	AMP; CAR; CKF; CXM; TE	Rough	II	6
A7	Chicken	1	54; 22.9	AMP; CAR; CKF; CXM; PRL; SAM; TE	Rough	II	6
A8	Chicken	1	54; 22.9	AMP; CAR; CKF; TE	Rough	II	6
A9	Chicken	1	54; 22.9		Rough	IV	6
A12	Chicken	1	54; 28.7		Rough	III	6
A23	Chicken	1	54; 45.5		Rough	III	UT
A10	Chicken	1	54; 28.7	AMP; CAR; C; PRL	Smooth	III	6
A11	Chicken	1	54; 28.7	AMP; CAR; CKF; PRL	Smooth	III	6
A13	Chicken	1	54; 28.7	AMP; CAR; CKF; CXM; PRL; SAM; TE	Smooth	III	6
A14	Chicken	1	54	AMP; CAR; CKF; CXM; PRL; TE	Smooth	III	6
A15	Chicken	1	54	AMP; CAR; CKF; PRL; TE	Smooth	III	6
A16	Chicken	1	54	AMC; AMP; CAR; CKF; CXM; PRL; SAM; TE	Rough	III	6
A17	Chicken	1	31.9	AMC; AMP; CAR; CKF; CXM; PRL; SAM; TE	Rough	III	35
A22	Chicken	1	54; 45.5	TE	Rough	IV	UT
A25, A26	Chicken	2	54; 45.5	TE	Rough	III	1

3.7.4. Statistical Analysis of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Chicken Isolates

Two main clusters were observed when the dendogram was constructed from the data of all the parameters tested for *S. enteritidis* chicken isolates (Figure 3.40)

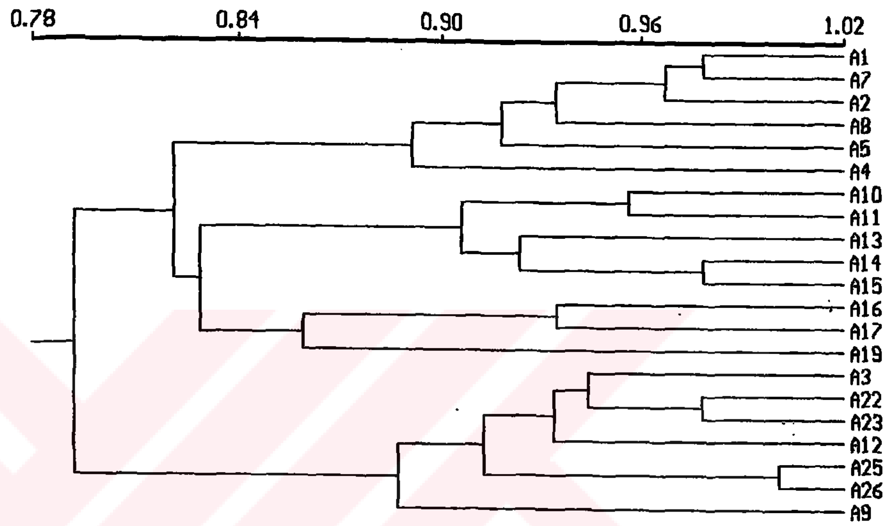


Figure 3.40. Dendogram constructed from the data of all the parameters tested for *S. enteritidis* chicken isolates

PCA resulted in 3 clusters (Figure 3.41). The isolate A17 was out of the clusters.

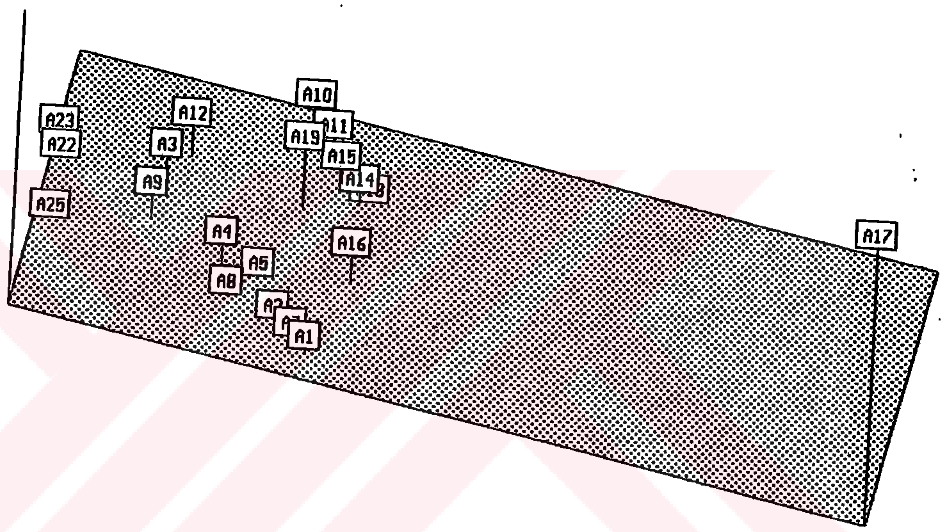


Figure 3.41. PCA of data for all the parameters tested for *S. enteritidis* chicken isolates in three dimensions

3.7.5. Comparison of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Egg Isolates

The comparison of antibiotic resistance, plasmid, LPS, PT and OMP profile of *S. enteritidis* egg isolates are tabulated in Table 3.28. 8 different profiles were identified for the ten *S. enteritidis* egg isolates. Chicken isolates with OMP-typeII outer membrane protein showed 6 different PTs. No direct relationship was found between plasmid profile and antibiotic resistance patterns. Although the isolates E9, E13 and E10 contained high molecular weight plasmids, they were not resistant. Smooth type LPS profile was expressed by only two (PT4 and PT20a) of the 10 strains.

Table 3.28. Comparison of antibiotic resistance, plasmid, LPS, OMP and PT profiles of *S. enteritidis* egg isolates

Strain Designation	Source	Number of Strains	Plasmid Profile in kb	Antibiotic Resistance Profile	LPS Profile	OMP Type	PT
E1, E4	Egg	2	54; 29.7; 16.5	TE	Smooth	II	4
E3	Egg	1	54	TE	Smooth	II	4
E7	Egg	1	54; 16.5; 2.2; 1.6	TE	Smooth	II	20a
E8	Egg	1	54; 29.7; 16.5	AMP; CKF; CXM; TE	Rough	II	9c
E12	Egg	1	54; 29.7; 16.5	AMP; CKF; CXM; TE	Rough	II	7
E9, E13	Egg	2	54; 29.7		Rough	II	4
E10	Egg	1	54; 29.7; 16.5		Rough	II	1
E11	Egg	1	54; 29.7; 16.5; 5.7; 4.5	TE	Rough	II	25

3.7.6. Statistical Analysis of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of *S. enteritidis* Egg Isolates

The dendogram constructed by using the antibiotic resistance, plasmid, LPS, PT and OMP profiles of *S. enteritidis* egg isolates resulted in a single main cluster (Figure 3.42).

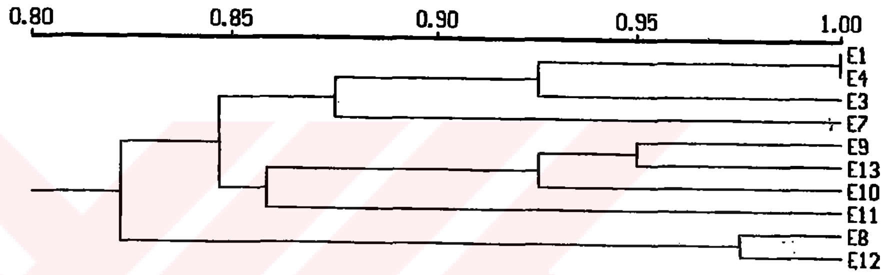


Figure 3.42. Dendogram constructed from the data from all the parameters tested for *S.enteritidis* egg isolates

On the other hand, PCA revealed the presence of 2 main clusters (Figure 3.43). The isolates E7 and E11 were out of the cluster.

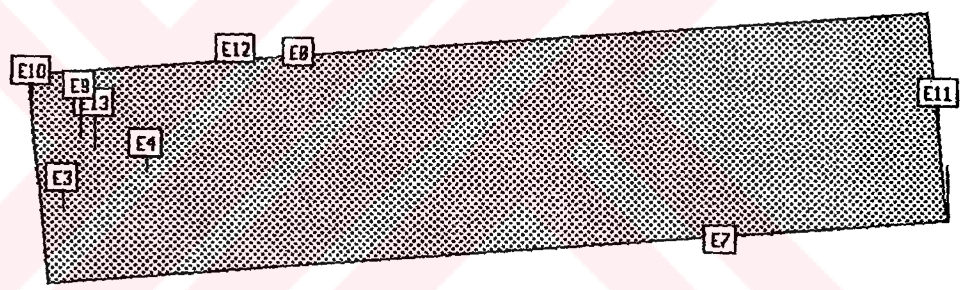


Figure 3.43. PCA of the data for all the parameters tested for *S. enteritidis* egg isolates in three dimensions

3.7.7. Comparison of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of the Members of *Enterobacteriaceae* Other Than *S.enteritidis*

Table 3.29 represents an overlook to the antibiotic resistance, plasmid, LPS, PT and OMP profiles of the enterics other than *S. enteritidis*. A direct reletionship between OMP types and LPS patterns could not be found. Likewise, there was no correlation between plasmid and antibiotic resistance patterns.

Table 3.29. Comparison of antibiotic resistance, plasmid, LPS, OMP and PT profiles of the enterics other than *S. enteritidis*

Strain Designation	Name of the Species	Source	Plasmid Profile in kb	Antibiotic Resistance Profile	LPS Profile	OMP Type	PT or Antigenic Structure
E2	*Salmonella kedougou*	Egg	1,6	TE	Smooth	II	I 13, 23: i: l, w
E5	*Salmonella*	Egg	54; 29.7; 16.5	TE	Rough	II	I O=rough: g, m: -
E6	*Enterobacter*	Egg	54; 29.7; 16.5	TE	Smooth	II	UT
A6	*Salmonella typhimurium*	Chicken	5.5; 4.9; 3.9; 3.5	TE	Rough	II	PT99
A18	*Salmonella ouakam*	Chicken	2.5; 1.8; 1.4; 1.2		Smooth	III	I 9, 46: z29
A20	*Salmonella typhimurium*	Chicken	5.5; 4.9; 3.9; 3.5	AMP; PRL; CAR; CXM; AZT	Rough	II	PT164
A21	*Salmonella ouakam*	Chicken		TE	Rough	III	I 9, 46: z29
A24	*Salmonella ouakam*	Chicken	54; 45.5	AZT; TE	Rough	III	I 9, 46: z29
H23	*Salmonella baildon*	Human		PRL; CXM; TE	Smooth	III	I 9, 46: a: e,n, x
H28	*Salmonella typhimurium*	Human	54; 28.4; 5.3; 4.3; 3.7; 3.5; 1.5		Smooth	II	UT
H29	*Salmonella typhimurium*	Human	54	AMP; PRL; CKF; CEP; SXT; SAM; AZT; TE; C	Smooth	II	PT104
H31	*Salmonella typhimurium*	Human	54	TE; C	Smooth	II	PT104
H32	*Salmonella havana*	Human	54; 28.4; 2.3; 1.9	AMP; PRL; CAR	Smooth	II	I 13, 23: f, g: -
H33	*Salmonella typhimurium*	Human	54; 4.8; 2.3; 2.1	TE	Smooth	II	PT104c
H38	*Salmonella agona*	Human	24,9	TE	Smooth	II	PT1
H49	*Salmonella typhimurium*	Human	54; 3.3; 2.8	AMP; PRL; CAR; CKF; CXM; SAM; AMC; TE; C	Smooth	II	PT104
H51	*Citrobacter freundii*	Human			Smooth	II	UT
H54	*Enterobacter*	Human	54; 27.5; 9.5	TE	Smooth	III	UT
H60	*Proteus*	Human		AMP; CKF; TE; C	Rough	IV	UT
H61	*Citrobacter*	Human	54	AMP; CKF; CEC; CXM; SAM; AMC; TE	Smooth	III	UT

*UT Untypable

3.7.8. Statistical Analysis of Antibiotic Resistance, Plasmid, LPS, PT and OMP Profiles of All *S. enteritidis* Strains From Different Sources

A dendogram was constructed by using antibiotic resistance, plasmid, LPS, PT and OMP profiles of all *S. enteritidis*. The dendogram resulted in 5 main clusters (Figure 3.44). In three of these clusters there was a high percentage of similarity between human and egg isolates. Only one cluster showed high percentage of similarity among isolates from all three sources. There was another cluster revealing high percentage of similarity between human and chicken isolates.

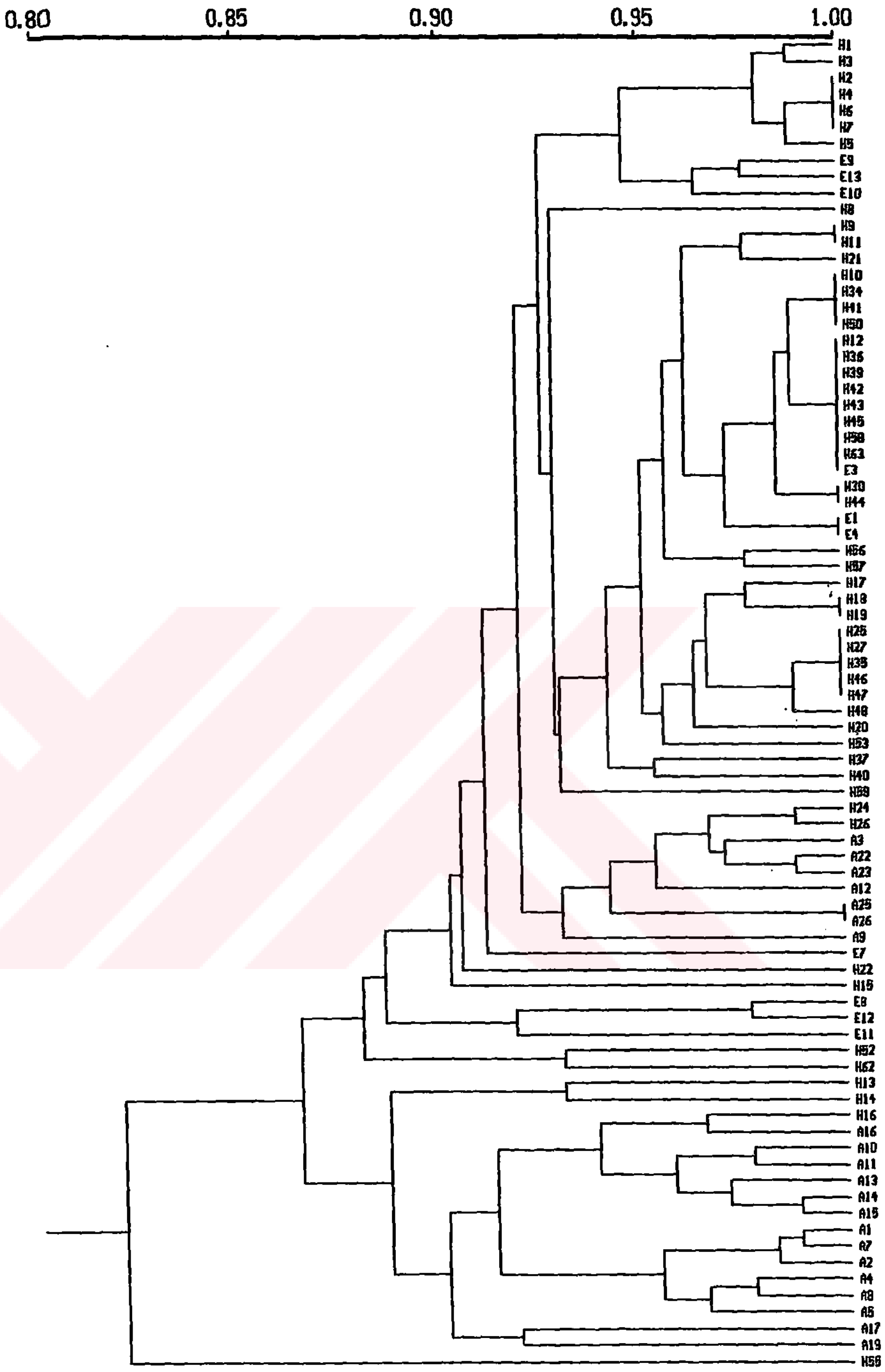


Figure 3.44. Dendogram constructed using the data of all the parameters tested for all *S. enteritidis* isolates of three sources

PCA resulted in a single big cluster (Figure 3.45), indicating that the strains from three different sources highly related with respect to the parameters tested. The isolates H53, H59 and H55 were out of the clusters.

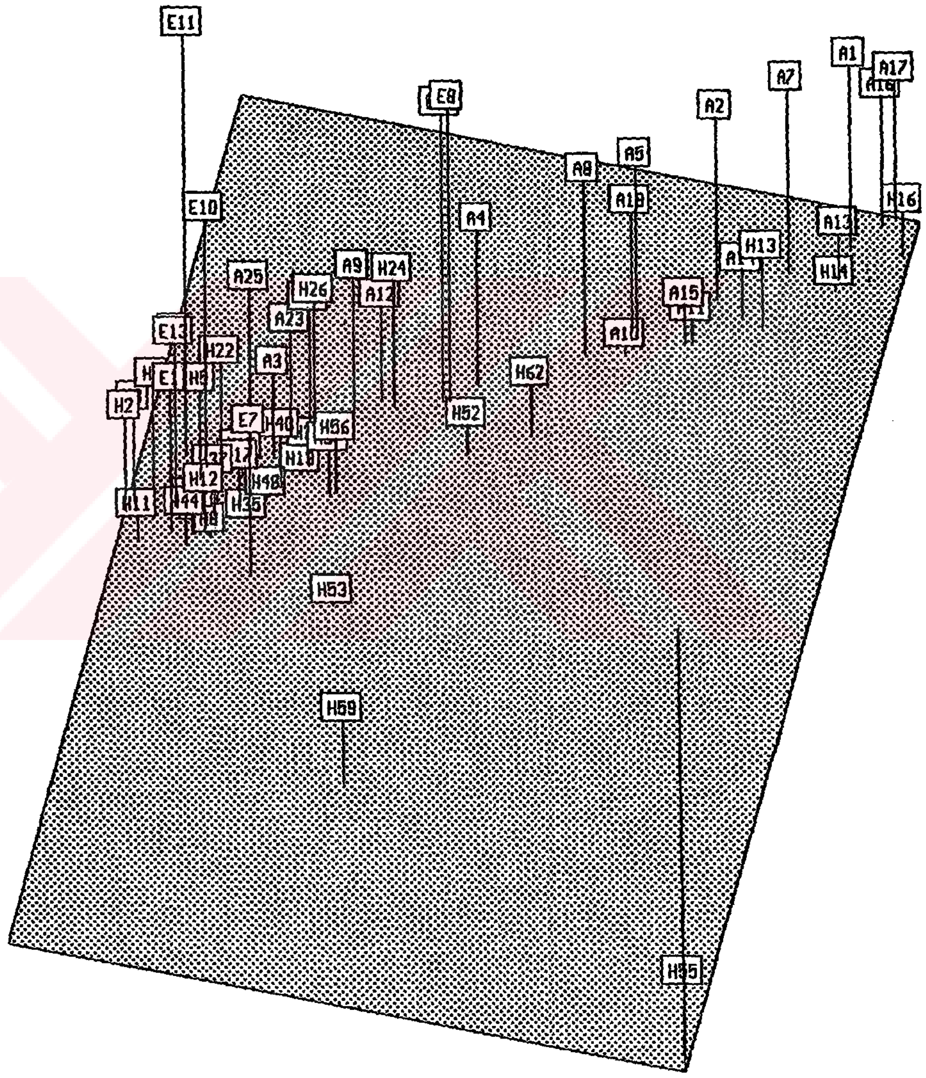


Figure 3.45. PCA of the data for all the parameters tested for *S. enteritidis* isolates from three sources

3.8. Plasmid Curing and Antibiotic Resistance, Plasmid, OMP, PT, and LPS Patterns of the Cured Isolates

The isolates A13, A14, E2, E7, H32 and H62 were used in curing experiments. Antibiotic-sensitive derivatives of the isolates were designated CA13, CA14, CE2, CE7, CH32 and CH62, respectively. The antibiotic resistance patterns of the strains are given in Table 3.30. It was found that the derivatives CE2, CE7, CH32 and CH62 have lost their resistance to all of the antibiotics. Although the isolate CA13 has lost the resistance to sulbactam and tetracycline antibiotics, the resistance to ampicillin, carbenicillin, cephalothin, cefuroxime and piperacillin antibiotics has been maintained. The isolate CA14 has become sensitive to the cephalothin, cefuroxime, sulbactam and tetracycline antibiotics while it was still resistant to ampicillin and carbenicillin.

Table 3.30 Antibiotic resistance patterns of the cured isolates

		ANTIBIOTICS																								
Strains	Source of the Strain	AK	AMC	AMP	AZT	CAR	CEC	CKF	CEP	CAZ	CRD	CXM	CIP	C	GM	IPM	OFX	PRL	CFM	SP	SAM	TE	TO	SXT	CD	MP
CE2	Egg																					S				
CE7	Egg																					S				
CA13	Chicken			R		R		R				R						R			S	S				
CA14	Chicken			R		R		S				S						R			S	S				
CH32	Human			S		S												S								
CH62	Human			S	S		S	S				S						S				S				

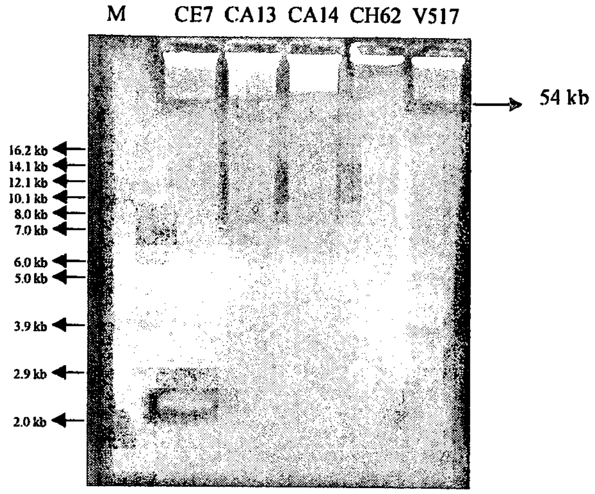
R Resistant CE2, CE7, CA13, CA14, CH32, CH62 (Cured derivatives)
S Sensitive E2, E7, A13, A14, H32, H62 (Parental strains)

AK	Amicasin-AK	CAZ	Ceftazidime-CAZ	PRL	Piperacillin-PRL	MP	Meropehem
AMC	Amoxicillin-AML+ Clavunalic Acid	CRD	Ceftriaxone-CRD	CFM	Cefixime-CFM		
AMP	Ampicillin	CXM	Cefuroxime-CXM	SP	Sulperazone-75 SP		
AZT	Aztreonam (Azactam)	CIP	Ciprofloxacin-CIP	SAM	Sulbactam Ampicillin-SAM		
CAR	Carbenicillin-Car	C	Chloramphenicol-c	TE	Tetracyclin-Te		
CEC	Cefaclor-CEC	GM	Gentamicin-GM-CN	TO	Tobramycin-NN		
CKF	Cephalothin-KF	IPM	Imipenem-IP	SXT	Trimethoprim Sulfamethoxazole		
CEP	Cefoperazone-CEF-CEP	OFX	Ofloxacin-OFX	CD	Cefodizime		

Table 3.31 represents a sum of plasmid profiles of the derivative strains. The isolates CA14 and CE2 did not loose any of the plasmids. On the other, hand the isolates CH32 and CH62 were found to lack all of the plasmids of their parental strains. The isolate CA13 lost the 28.7 kb plasmid while the isolate CE7 lost its low molecular weight plasmids of 16.5 and 2.2 kb. The plasmid profiles of these strains are shown in Figure 3.46.

Table 3.31. Plasmid profiles of the strains after curing

Strain Designation	Species	Source	Plasmids in kb (Before curing)	Plasmids in kb (After curing)
CE2	*Salmonella kedougou*	Egg	1.6	1.6
CE7	*Salmonella enteritidis*	Egg	54; 16.5; 2.2; 1.6	54; 1.6
CA13	*Salmonella enteritidis*	Chicken	54; 28.7	54
CA14	*Salmonella enteritidis*	Chicken	54	54
CH32	*Salmonella havana*	Human	54; 28.4; 2.3; 1.9	-
CH62	*Salmonella enteritidis*	Human	54	-



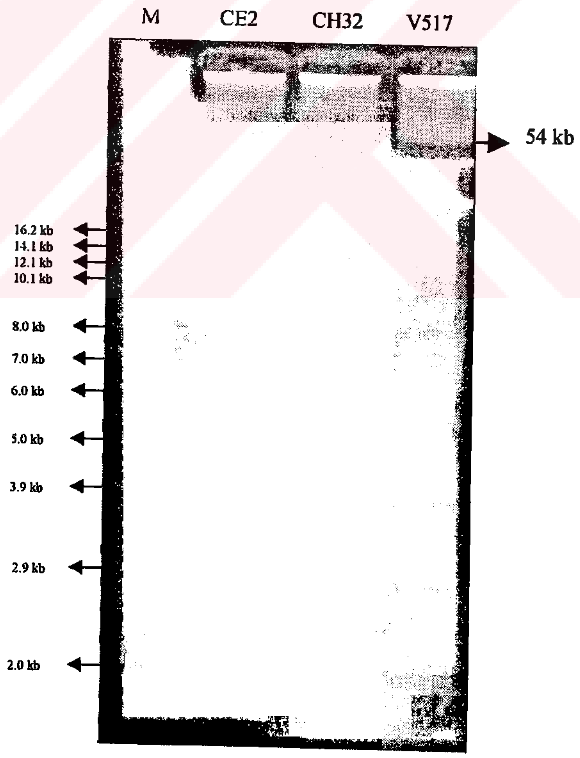
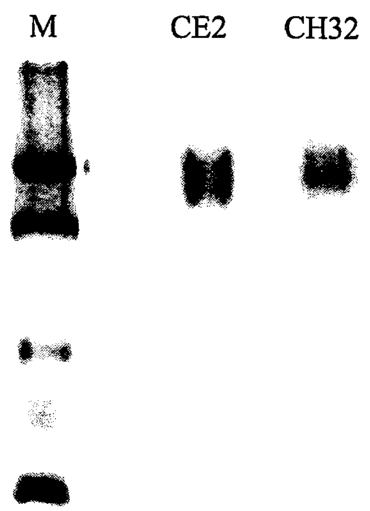


Figure 3.46. Plasmid profiles of the strains after curing

LPS profiles of the cured isolates are given in Table 3.32. All the cured isolates have lost their ability to express long-chain LPS and converted to rough type (Figure 3.47).

Table 3.32. LPS profiles of the strains after curing

Strain Designation	Name of the Species	Source	LPS Profile (Before curing)	LPS Profile (After curing)
CE2	*Salmonella kedougou*	Egg	Smooth	Rough
CE7	*Salmonella enteritidis*	Egg	Smooth	Rough
CA13	*Salmonella enteritidis*	Chicken	Smooth	Rough
CA14	*Salmonella enteritidis*	Chicken	Smooth	Rough
CH32	*Salmonella havana*	Human	Smooth	Rough
CH62	*Salmonella enteritidis*	Human	Smooth	Rough



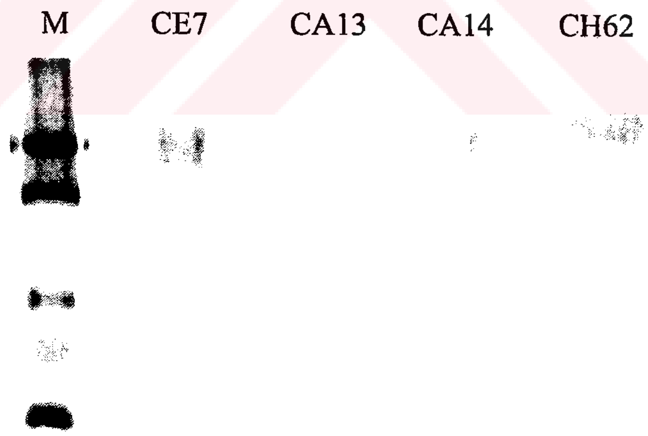


Figure 3.47. LPS profiles of the strains after curing

Table 3.33 shows the phage typing of the strains after curing. The phage types of the two isolates have changed after curing. The CA13 isolate which belongs to PT6 has been converted to PT21 and CE7 which is originally PT20a has become PT20 variant. The phage types of the other isolates has not changed.

Table 3.33. PT profiles of the strains after curing

Strain Designation	Name of the Species	Source	PT or Serology (Before curing)	PT or Serology (After curing)
CE2	*Salmonella kedougou*	Egg	I 13, 23: l,w	I 13, 23: l,w
CE7	*Salmonella enteritidis*	Egg	20a	20variant
CA13	*Salmonella enteritidis*	Chicken	6	21
CA14	*Salmonella enteritidis*	Chicken	6	6
CH32	*Salmonella havana*	Human	I 13, 23: f,g	I 13, 23: f,g
CH62	*Salmonella enteritidis*	Human	4	4

It was found that OMP type changes has occurred only in the isolate CA13, which has been converted from OMP-typeIII to OMP-typeII. (Figure 3.48)

Table 3.34. OMP profiles of the strains after curing

Strain Designation	Name of the Species	Source	OMP Profile (Before curing)	OMP Profile (After curing)
CE2	*Salmonella kedougou*	Egg	II	II
CE7	*Salmonella enteritidis*	Egg	II	II
CA13	*Salmonella enteritidis*	Chicken	III	II
CA14	*Salmonella enteritidis*	Chicken	III	III
CH32	*Salmonella havana*	Human	II	II
CH62	*Salmonella enteritidis*	Human	III	III

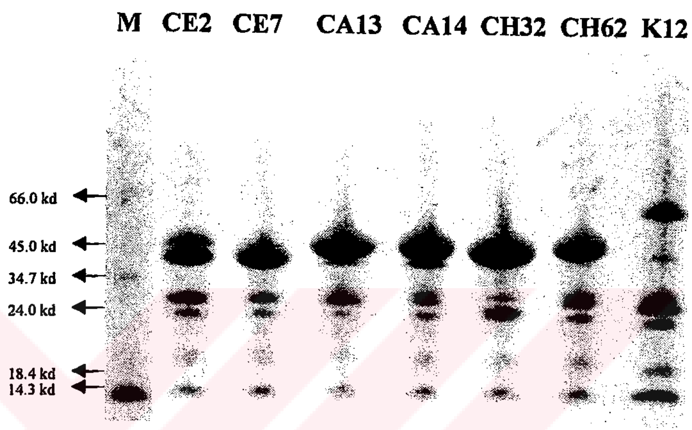


Figure 3.48. OMP profiles of the strains after curing

CHAPTER 4

DISCUSSION

Salmonellosis in the United States results in considerable morbidity. Over 40.000 isolates of *Salmonella* are reported each year. However, these reports represent only a small fraction of the number of cases that actually occur. Infection may not be suspected, cultured, diagnosed, or reported for a variety of reasons. Community surveys during outbreaks suggest that the proportion of actual infections reported are between 1 in 10 and 1 in 100 (Ryan *et al.,* 1985). Thus, between 40.000 and 4.000.000 cases may occur annually.

Salmonellosis is also associated with considerable financial cost. Animal producers face costs from illness and death of animals, restaurants and industry incur costs from lost business and lawsuits, and patients experience direct and indirect costs. When the total costs of unreported cases and the costs to industry and business are included, salmonellosis may cost billions of dollars each year (Archer and Kvenberg, 1985).

The significance of antimicrobial resistance is most obvious in impact on treatment of human infections, but there are other less obvious and perhaps more important effects. Most *Salmonella* infections do not require treatment.

Antimicrobials are not indicated for uncomplicated gastroenteritis since treatment does not reduce the duration or severity of symptoms, may prolong convalescence and the carrier state, and may result in the emergence of resistant organisms (Aserkoff and Bennett 1969). Some physicians treat infants and elderly persons who have uncomplicated gastroenteritis, attempting to prevent complications such as local infections or bacteremia, which occur most frequently in these age groups. In the CDC (Centre for Disease Control and Prevention) prospective studies, 40% of patients with salmonellosis were treated with antimicrobial drugs (Cohen and Tauxe 1986).

Another impact of antimicrobial resistance is through the recently recognized association between drug resistant salmonellae and the routine clinical use of antimicrobials for infections other than salmonellosis. There are three aspects of this association: i) Antimicrobial-resistant *Salmonella* infections can complicate antimicrobial treatment of other infections. ii) Prior antimicrobial therapy allows fever numbers of antimicrobial-resistant *Salmonella* to cause symptomatic infections. iii) An increase in the proportion of salmonellae that are antimicrobial-resistant will increase the overall frequency of salmonellosis. The increase in chloramphenicol resistance in *Salmonella* suggests that the use of this drug in animals may be leading to increased resistance in human *Salmonella* infections. During antimicrobial therapy in humans, *Salmonella* may acquire transferable resistance factors from other enteric flora. The developing world has attracted considerable attention as a potential source for antimicrobial resistance of various human pathogens since these countries have well-recognized problems with antimicrobial-resistant bacteria. Several authors have suggested that antimicrobial resistance in *Salmonella* is the result of antimicrobial use within the hospital (Datta and Pridie, 1960; Abbott *et al.*, 1980; Opal *et al.*, 1989). Although the hospital may be an important source for resistant *Salmonella*, there is little evidence that this is true in developing world.

In our study, multi-resistance was uncommon in *S. enteritidis* human and egg isolates. Multiple resistance has also been shown to be extremely rare in *S. enteritidis* isolates form England and Wales (Threalfall *et al.*, 1993), and also from United States (Lee *et al.*, 1994). On the other hand multi-resistance was found to be common in this study among *S. enteritidis* chicken isolates from Turkey. This may be due to the introduction of restrictive measures on antibiotic usage in farming. Overall, our study revealed that strains of egg origin were least multi-resistant, while those isolated from chicken were more multi-resistant than those from human sources. These findings are somewhat different from those reported for most other European countries, including England, Germany and Netherland (Brown *et al.*, 1984; Hefferman, 1991; Threlfall *et al.*, 1993).

Animals are fed antimicrobials to prevent and to treat diseases and to enhance growth. The use of low doses of antimicrobials is frequently referred to as subtherapeutic use because the amount of antimicrobial administered is less than that used to treat specific illnesses. The use of subtherapeutic doses as growth enhancers, which accounts for much of the antimicrobial usage, produces effects on animals that are similar to the effects seen in humans who receive antimicrobials for salmonellosis. Animals fed subtherapeutic doses of antimicrobials develop resistant organisms. That might explain why tetracycline resistance was common in *S. enteritidis* strains isolated from human, chicken and egg sources in our study. In contrast to control animals, animals fed subtherapeutic doses of antibiotic excrete resistant organisms in their feces in large numbers for longer periods and also transmit the organisms to other animals. Because large number of animals have longer and more constant exposure to subtherapeutic antimicrobials than to therapeutic ones, such use may have a greater impact then therapeutic use on the rate at which resistants strains of bacteria develop and persist in the environment. Antimicrobial use in animals also has other effects similar to those observed in humans.

When compared to the resistance reports from some other countries (Munoz *et al.*, 1993; Fahoudi-Moghaddam *et al.*, 1990, Varaiki *et al.*, 1996), the rate of drug resistance has been shown to be rather low in Turkey. A previous study conducted between 1989 and 1990 revealed that the incidence of resistance of *Salmonella* to older antibiotics (ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole and tetracycline was around 85% (Yıldırmak, 1990), a percentage significantly higher than those determined by Arman in 1993 (22-23%), and by Yıldırmak and his colleagues in 1998 (8-19%). The decline in resistance to these antibiotics has been most likely due to the widespread clinical use of quinolones and third-generation cephalosporins in Turkey since 1990. Salmonella isolates had been susceptible to third-generation drugs before this year (Yıldırmak, 1990), thus the use of these drugs should have also accounted for the emergence of extended-spectrum β-lactamases producing pathogens resistant to third-generation cephalosporins (Yıldırmak *et al.*, 1998). In our study, in contrast to these findings obtained from the isolation of *S. typhimurium*, *S. enteritidis* human, chicken and egg isolates showed high percentage of resistance to tetracycline and ampicillin although the resistance to chloramphenicol and trimethoprim-sulfamethoxazole was very low.

Patients with bacteremia, meningitis, or other extraintestinal infections require effective antimicrobial treatment . Chloramphenicol had been the drug of choice for decades because it was effective in treating typhoid fever and because the frequency of chloramphenicol resistance *Salmonella* had been low. Other effective drugs included were ampicillin and trimethoprim-sulfamethoxazole. Ciprofloxacin is often used to treat serious salmonella infections now, because of its rapid bactericidal action (Piddock *et al.*, 1990), and because it has been shown to be clinically effective and well-tolerated (Asperilla *et al.*, 1990). There are very few reports of resistance to ciprofloxacin in salmonellae (Piddock *et al.*, 1993; Umasankar *et al.*, 1992; Pers *et al.*, 1996).

On the other hand, despite the extensive usage of quinolones, none of the isolates were found to be resistant to these antibiotics. Our isolates were also found to be sensitive to aminoglycosides. The general decrease in resistance to these drugs might also be explained by the ability of them to eliminate "R" plasmids and to inhibit conjugal transfer (Weisser, 1987). If the frequency of resistance to quinolone and aminoglycoside type antibiotics rises, the choice of agents that can be used to treat patients becomes more limited, and the likelihood increases that initial therapy, chosen before sensitivity data on the infecting organism are available, will fail very sick patients. Such a fetal case in a patient infected with quinolone-resistant *Salmonella* in the United States was reported by Heriskstad and his colleagues in 1997.

In our study, the statistical analysis of the antibiogram data obtained from *S. enteritidis* isolates from three sources showed that chicken and egg isolates showed high percentage of similarity to human isolates (Figure 3.6), thus suggesting a common origin for these strains in view of the hypothesis that resistance originating in animal strains can be transmitted to infected humans (Chaslus-Dancla *et al.*, 1991; Holmberg *et al.*, 1984a; Homberg *et al.*, 1984b; O'Brien *et al.*, 1982). Furthermore, the transmissibility of antimicrobial resistance and virulence factors through transmission cycle may contribute to the development and dissemination of other pathogenic *Salmonella* serotypes (Homberg *et al.*, 1984b). Accordingly, the antibiogram of the other enterics used in our study gave similar results with *S. enteritidis* isolates.

Plasmids have been shown to be potential strain markers (Baird *et al.*, 1985; Frost *et al.*, 1989; Taylor *et al.*, 1982). Plasmid profiling is therefore a preferable method to differentiate strains from each other and to identify a strain in a "cause and effect" relationship (Riley and Cohen, 1982). Plasmid profiling relies on the analysis of genetic content, rather than on the phenotypic characteristics of the microorganism which is usually considered in most other typing schemes (Manning *et al.*, 1989). This reliance on genetic content has rendered the conventional epidemiological markers of little value in differentiating many strains in epidemic relationships (Bezanson *et al.*, 1983). The bacterial plasmid profile for a given strain could be specific during a limited interval and in a limited area.

The use of plasmid profile analysis has proven to be very discriminating tool in linking isolates in epidemiological investigations, such as *S. newport, S. munster* and *S. typhimurium* (Bezanson *et al.*, 1983; Holmberg *et al.*, 1984; Riley and Cohen, 1982). In some cases, such as the *S. muenchen* outbreak involving marijuana in a multistate outbreak, the presence of the plasmids in the epidemic strain was the only evidence that linked these isolates together (Taylor *et al.*, 1982). It is important to note that many of the conventional methodologies used on outbreak-related isolates and unrelated isolates demonstrated no differences in antibiograms. Plasmid profiles presented the only firm evidence that the isolates were linked. (Jones *et al.*, 1982;). Others have used plasmid profiling and plasmid endonuclease fingerprinting to investigate the molecular epidemiology of antibiotic resistance in *Salmonella* from animals and humans in the United States (Bezanson *et al.*, 1983; Godson and Vapnek, 1973; Nagaraja, 1989; O'Brien *et al.*, 1982; 38).

Current evidence has indicated that high molecular weight (HMW) plasmids are needed for the virulence of several serotypes of salmonella which are capable of producing disesase in humans and animals. These HMW plasmids have been shown to be present in *S. typhimurium* (Jones *et al.*, 1982; Spratt *et al.*, 1973), *S. dublin* (Manning *et al.*, 1986; Terakado *et al.*, 1983), *S. enteritidis* (Nakamura *et al.*, 1985), *S. pullorum* (Barrow and Lovell, 1988), and *S. gallinarum* (Barrow *et al.*, 1987). The loss of these plasmids has resulted in a reduction or loss of virulence in these serotypes (Jones *et al.*, 1982). It has been demonstrated that the 54 kb virulence associated (VA) HMW plasmid makes a definitive contribution to the expression of virulence in *S. enteritidis* (Frost *et al.*, 1989; Helmuth *et al.*, 1985; Nakamura *et al.*, 1985; Tacket, 1989; Tenover, 1985). In the present study, it was also revealed that a 54 kb HMW was harbored by 94.11% of the human, 90.47% of the chicken and all of the egg isolates. Because these HMW plasmids exist to such a high degree in isolates of *S. enteritidis*, the HMW plasmid alone cannot be used as an epidemiological marker unless their identity can be verified through restriction analyses. Still, most isolates of *S. enteritidis* contain multiple antibiotic resistance "R" plasmids, and the complement of these plasmids can serve as a strain marker in an epidemiological investigation. The 54 kb plasmid was harbourd also by other enterics used in our study, but the percentage of such isolates was not as high as in the *S. enteritidis* isolates. On the other hand plasmid profile data indicated no strict association between plasmid patterns and antibiotic resistance patterns. The heterogeneity of the plasmid profiles is thought to reflect the sporadic and non-repetitive nature of the isolates. On the other hand, the cured isolates displayed antibiotic resistance patterns different than the parental strains. Although the isolate E2 did not loose any of the resident plasmid after the curing treatment, it became tetracycline sensitive. Tetracycline resistance is thus thought to be determined by a chromosomal gene. That is accounted for the chromosomal origin of that property. The cured isolate CA13 lacked the plasmid of 28.7 kb which accounted for the loss of sulbactam ampicillin resistance.

A broad range of identification methodologies has been developed to identify the relatedness of *Salmonella* isolates in an epidemiological investigation. Reliance on methods utilizing the genetic endowment of an organism, such as plasmid profiling, is preferable, because of the existence of variability in plasmid size and stability in a given strain of *S. enteritidis* (Brunner *et al.*, 1983). It can be concluded that *S. enteritidis* isolates can be characterized by establishing their plasmid profile.

In our study, 30 of the human (H10, H12, H13, H14, H17, H18, H19, H20, H24, H25, H26, H27, H30, H34, H35, H36, H39, H41, H42, H43, H44, H45, H46, H47, H48, H50, H57, H58, H62, H63), 5 of the chicken (A3, A4, A14, A15, A16) and an egg isolate (E3) contained the same plasmid profiles. One half of the *S. enteritidis* isolates contained two or more plasmids. The similar results were obtained by Bichler *et al.*, in 1994. We observed that human isolates from Ankara and Bursa region and the chicken isolates from the same regions of Turkey showed high percentage of similarity in plasmid profiles. The chicken isolates from these regions appeared to be the least diverse with respect to the plasmid profiles. In this regard, our findings do not fit to those of Bichler and his colleagues reported in 1994.

On the other hand, plasmids of similar size or even identical plasmid profiles do not necessarily lead to an establishment of epidemiological association between two strains of *S. enteritidis* . A further degree of differentiation can be achieved by restriction endonuclease fingerprinting of plasmid DNA. The fingerprint is especially useful when discriminating between plasmids of similar molecular size (Threlfall and Frost, 1990). As this technique also provides a measure of plasmid relatedness, it can be useful when tracing the evolutionary origin of plasmids and of their host strains (Platt *et al.*, 1986; Threlfall and Frost, 1990).

The traditional methods of strain identification based on serotyping and phage typing have been used in epidemiological study of salmonella for many years and despite the increasing use of typing methods based on molecular biological techniques, continue to provide the basis for the study of salmonella epidemiology (Brown *et al.*, 1999). Phage typing has been mainly used to differentiate isolate within serotypes of *S. typhi* (Anderson and Williams, 1956), *S. typhimurium* (Callow, 1959), *S. virchow* (Chambers *et al.*, 1987), *S. hadar* (De Sa *et al.*, 1980) and *S. enteritidis* (Ward *et al.*, 1987). The strains responsible for the dramatic increase in the incidence of human cases of salmonellosis caused by *S. enteritidis* in many countries around the world was identified by the use of phage typing. It was found that human salmonellosis was caused predominantly by strains of PT4 in Europe (Threlfall *et al.*, 1989), and PT8 and PT13a in the USA and Canada (Rodrique *et al.*, 1992; Khakhria *et al.*, 1991). Phage typing of *S. enteritidis* has been used to provide the basis for epidemiological analysis and as much as 44 distinct phage types are now recognized (Threlfall and Chart, 1993).

In our study, PT4 was shown to predominate in *S. enteritidis* human and egg isolates. On the other hand, PT6 was common in *S. enteritidis* chicken isolates. The *S. enteritidis* isolates from three sources generally belonged to the phage types PT1, PT4, PT6 and PT7 and pointed to an epidemiological connection between the isolates from human, poultry and eggs. Our findings were similar those of Coyle *et* *al*., (1988), Frost *et al*., (1988) and St. Lois *et al*., (1988). The finding that PT4 was the predominat phage type in strains of *S. enteritidis* in Turkey. It was also reported previously study by Erdem and her colleagues in 1994. On the other hand, the isolates with the same phage types had different plasmid and antibiotic resistance profiles. This indicated that plasmid profiles can not be used in prediction of phage types in *S. enteritidis*. In this respect, our results are in accordance with those of Bichler and his colleagues (1994). Antimicrobial susceptibility patterns were not as useful for typing *S. enteritidis* as were plasmid profile typing and phage typing, since most outbreak and sporadic isolates were non-resistant as reported by Holmberg *et al*., in 1994 and by Rodrique *et al*., in 1992. Our results showed that plasmid profile typing was not as discriminatory as phage typing for the primary subdivision of *S. enteritidis*. Ideally, plasmid profile determination should be used as an adjunct to phage typing in support of epidemiological investigations. Nevertheless, plasmid profile typing may be a simple technique to provide limited strain differentiation for laboratories which do not have access to phage typing. The of 54 kb plasmid was very common among the *S. enteritidis* strains and the strains belonged to only six phage types. Thus, the possession of 54 kb plasmid is not a very discriminatory feature to differentiate between *S. enteritidis* strains and analysis of plasmid profile alone would be of limited value. Phage typing subdivides the large group of strains with a single 54 kb plasmid into six major groups and is therefore regarded to be more discriminatory. Threlfall *et al*., (1989) and Morris *et al*., (1992) suggested that both phage typing and plasmid profile typing should be determined for an effective discrimination between the isolates.

Although phage typing has remained the method of choice being rapid, reproducable, discriminatory and cost effective (Threlfall and Frost, 1990), there have been reports of phage type conversion caused by the acquisition or the loss of plasmid DNA in *S. typhimurium* (Anderson *et al.*, 1973; Platt *et al.*, 1987), and *S. typhi* (Datta and Richards, 1981). The conversion of *S. enteritidis* PT4 to PT24 following acquisition of an Inc*N*, drug resistance plasmid was reported by Frost and his colleagues (1989). It was recently shown that PT4 could be converted to PT8 following lysogenization with phage 2 from the *S. enteritidis* typing scheme (Rankin and Platt, 1995).

The isolates E2, E7, A13, A14, H32 and H62 exposed to curing treatment lost of some of the resident plasmids and phage type conversion was detected in the isolates CE7 and CA13. The phage type conversion in CA13 from PT6 to PT21 has been associated with the loss of 28.7 kb plasmid. On the other hand, the change in the phage type of the strain (CE7 from PT20a to PT20variant) was accompanied by the loss of two plasmids of 16.5 and 2.2 kb. Although all the plasmids of the isolates CH32 and CH62 were cured, no phage type conversion was observed for these strains.

The main advantage of phage typing is that as much as 40 strains can be tested in a day. The phage typing system of Ward and her colleagues (1987) is reproducible, stable, internationally standardized, fast, and inexpensive. Propagation of the phages and determination of their distributions are done at the WHO International Center for Enteric Phage Typing, London England. Isolates from the

United States have been over 96% typeable by using this system. However, the phages are not available to all laboratories. In addition, a separate phage typing system is usually required for a specific species or serotype whereas a given molecular technique can generally be adapted for a variety of organisms.

Lipopolysaccharides (LPS), a major component of the surface, has received much attention in *Salmonella* species and its chemical composition biosynthesis, genetic regulation, and immunochemistry have been well characterized (Hitchcock and Brown, 1983). Current techniques for classifying *S. enteritidis* isolates have not resulted in a reliable method for correlating virulence and egg contamination, progress in defining the molecular events leading to outbreaks in people has been slow. One investigation of *S. enteritidis* virulence showed that variant isolated from a single parental culture had different abilities to contaminate eggs and to be recovered from the spleens of chicks, and these variants were determined to have different amounts of LPS O-antigen linked to core (Petter, 1993). Since a central factor in determining the virulence of gram-negative organisms is the structure of LPS (Brade *et al.*, 1988; Jimenez-Lucho and Leive, 1990; Kusumoto *et al.*, 1989; Raetz *et al.*, 1991; Rick, 1987), we detected LPS structure by using a simple method which is based on polyacrylamide gel electrophoresis (PAGE) to interpret differences in the migration patterns of discrete sugar moieties (Dubray and Bezard, 1982; Duchesne *et al.*, 1988; Helander *et al.*, 1992; Klena *et al.*, 1992; Kraus *et al.*, 1988; Palva and Makela, 1980; Tsai and Frasch, 1982). Analysis of bichemically characterized LPS by SDS-PAGE has revealed that the O-specific polysaccharides, core oligosaccharides, and Lipid A content contribute to the morphological heterogeneity of SDS-PAGE LPS profiles (Goldman and Lieve, 1980; Jann *et al.*, 1975; Munford *et al.*, 1980; Palva and Makela, 1980).

Rigorous analyses of LPS by SDS-PAGE have been hampered by the fact that LPS does not stain well with carbohydrate stains (periodic acid-Schiff Alcian Blue methods) (Segrets and Jackson, 1972; Corjo *et al.*, 1991). In the past, intrinsic radiolabels (Goldman and Leive, 1988; Munford *et al.*, 1980; Palva and Makela, 1980), carbohydrate dye (Procion red) (Anderson *et al.*, 1971; Jann *et al.*, 1975;), Periodic acid-Schiff stain (Munford *et al.*, 1980; Russell and Johnson, 1975), ethidium bromide (Kıdo *et al.*, 1990) and silver stain (Hitchcock and Brown, 1983) have been used to visulize LPS in polyacrylamide gels. In the present study, we used silver staining for detection of LPS after electrophoresis.

Fifty of the 82 *S. enteritidis* isolates (61%) contained smooth LPS as they gave typical ladder-type patterns which extended from the bottom to the top part of the gel. In the rest of the strains, only core LPS was present. It was remarkable that all four PT7 isolates from human and egg sources were rough and 4 PT8 strains from human sources were smooth. In this respect our results were similar to those of Chart and co-workers (1991) who showed that *S enteritidis* PT7 strains do not express long-chain LPS. Another observation was that strains of *S. enteritidis* belonging to PTs 4, 6, 8, 13 and 24 have been associated with poultry and poultry products (Chart *et al.*, 1993). In our study majority of *S. enteritidis* human isolates belonged to these PTs, suggesting that the possible origin of these organisms was poultry and poultry products.

The ability of strains of *S. enteritidis* to express smooth LPS has been shown to be the major virulence factor (Chart *et al.*, 1989a). In our study, the majority of the *S. enteritidis* human isolates (80.4%) showed smooth LPS. This was as expected since the isolates were obtained from patients with gastroenteritis.

In contrast to the report of Chart and Rowe (1991), there was no correlation between plasmid and LPS profiles. *S. enteritidis* PT4 has been shown to convert spontaneously to PT7 with complete loss of LPS expression (Chart *et al.*, 1989b). Plasmid curing in our study revealed that all the cured isolates expressed rough LPS. On the other hand, as mentioned earlier, only two of the isolates (A13 and E7) had converted PTs upon treatment for curing and both of the isolates contained 54 kb high molecular weight plasmid. The loss of LPSs may result from mutations affecting anyone of several specific steps in LPS biosynthesis (Rick, 1987). Strains of *S. enteritidis* belonging to PT4 having long-chain LPS and carrying a plasmid of 38 Mda was shown to be highly virulent for BALB/c mice (Chart *et al.*, 1989a). Chart and his colleagues (1989b) also showed that strains of *S. enteritidis* PT4, loss of 38 Mda plasmid or the inability to express LPS resulted in a million-fold decrease in virulence for mice. The virulence mechanisms encoded by the 38 Mda plasmid have not been fully elucidated (Chart and Rowe 1991).

The characteristic migration patterns of outer membrane proteins during SDS-PAGE has been used for subdividing *Neisseria meningitidis* (Frasch *et al.*, 1976), *Haemophilus influenzae* (Barenkamp *et al.*, 1981; Loeb and Smith, 1980), *E. coli* (Overbeeke and Lugtenberg, 1980; Achtman *et al.*, 1983), *Yersinia ruckeri*

(Davies, 1991), *Salmonella* serotypes (Helmuth *et al.*, 1985) and *S. enteritidis* (Poppe *et al.*, 1993). Because different migration patterns of outer membrane proteins have been observed with isolates such an analysis might have been expected to yield information on the relatedness of these isolates.

Sarkosyl extraction was chosen as the method for isolating the OMPs because it is relatively quick, reproducible, straightforward and has a widespread use (Barenkamp *et al.*, 1981; Buckley *et al.*, 1981; Blaser *et al.*, 1983; Murphy *et al.*, 1983; Odumeru *et al.*, 1983; Aoki and Holland, 1985; Rapp *et al.*, 1986; Davies, 1991; Poppe etal., 1993). Although the separation of cytoplasmic and outer membranes is sometimes carried out by isopycnic gradient centrifugation to avaoid the use of detergents. This method is cumbersome and time consuming (Stull *et al.*, 1985) and would not be suitable for studies of large numbers of isolates (Davies, 1991).

In our study, the *S. enteritidis* isolates contained OMPs with the molecular weight ranging between 25.56 kDa and 34.61 kDa. This finding differred from those of Poppe and his colleagues (1991) who found that *S. enteritidis* strains generally contained OMPS of 42, 40 and 37 kDA. On the other hand , Chart and Rowe (1991) showed that the *S. enteritidis* strains P132344, P132344/1 and P131245 contained three major OMPs of 33, 35 and 36 kDa. The present work revealed that the majority of human isolates (68.62%) and all of the egg isolates contained OMPs with the molecular weight of 30.65 and 34.61 kDa. On the other hand, majority of the chicken isolates (52.38%) carried OMPs of 27.15, 30.65 and 34.61 kDa. Another different

OMP profile was obtained from the chicken isolates A9, A22 and one of the other enteric isolate H60 (*Proteus* sp.) which contained OMPs of 25.56, 27.15, 30.65 and 34.61 kDa. 30.65 and 34.61 kDa OMPs were also common among the other enterics used in our study.

Variation occurs in the expression of peptidoglycan-essociated proteins (PAP) according to the phase of the growth cycle as suggested by Davies (1991). He showed that all isolates possessed a PAP in the MW range 34.7 to 45 kDa, although the expression of this protein varied according to the phase of growth. The MW range of PAPs was 36.5 to 40.4 kDa according to the undertaken study done by Davies (1991) who also showed that these proteins form stable complexes with peptidoglycan in 2% SDS at 60°C and below, but are dissociated from peptidoglycan at temperatures above this. It might be a possible explanation for the MW range of OMPs in the present study. PAPs form hydrophilic pores through the OM and are hence known as porins (Nakae, 1976; Lugtenberg *et al.*, 1977). 64.63% of the 82 *S. enteritidis* isolates obtained from Ankara and Bursa region were of OMP-typeII.

As opposed to the study done by Helmuth and his colleagues (1985), we did not find any correlation among plasmid content, LPS profile and OMP types. The lack of correlation among these parameters were also reported by Chart and Rowe (1991).

CHAPTER 5

CONCLUSION

- None of the *S. enteritidis* isolates from human, chicken and egg sources showed resistance to aminoglycoside, quinolone and carbapenem type antibiotics

- Tetracycline resistance was common in *S. enteritidis* isolates from all these three sources.

- Multi-resistance was common in *S. enteritidis* chicken isolates.

- Human isolates displayed the greatest number of different plasmid profiles while the smallest number of different plasmid profiles was obtained from chicken isolates.

- The 54kb high molecular weight plasmid was harbored by 94.11% of the human, 90.47% of the chicken and all of the egg isolates. A plasmid of same size was also detected in 55% of the nonenteritidis enteric strains.

- A specific plasmid profile common to some of the isolates might be useful in establishing causal relationships. The chicken isolates A3, A4, A14, A15, A16 and the egg isolate E3 were found to be identical to the human isolates H10, H12, H13, H14, H17, H18, H19, H20, H24, H25, H26, H27, H30, H34, H35, H36, H39, H41, H42, H43, H44, H45, H46, H47, H48, H50, H57, H58, H62 and H63.

- Ten phage types (PT1, PT4, PT6, PT7, PT8, PT9c, PT13a, PT20a, PT25, and PT35) were identified within 82 *S. enteritidis* strains.

- Phage type 4 (PT4) was predominating (53.65%) which was followed by PT6 (21.95%). None of the *S. enteritidis* egg isolates belonged to PT6.

- *S. enteritidis* isolates from humans, chickens and eggs belonging to the phage types PT1, PT4, PT6 and PT7 pointed to a possible association of epidemiological connection of these isolates.

- Antimicrobial susceptibility patterns were as useful for typing *S. enteritidis* as were plasmid profile typing and phage typing.

- 60.97% of the 82 *S. enteritidis* isolates expressed long-chain LPS giving a characteristic "ladder" pattern (smooth). The majority of the human isolates showed smooth type LPS although the majority of chicken and egg isolates carried rough LPS. All of the PT7 isolates were rough while all the PT8 isolates were smooth.

- 64.63% of the 82 *S. enteritidis* isolates contained OMPs with the molecular weights ranging from 34.61 to 30.65 kDa.

- There was not a direct correlation between the plasmid content, LPS and OMP profiles of the isolates.

- The dendogram established using the whole data (antibiotic resistance, plasmid, LPS, PT and OMP profiles of *S. enteritidis*) revealed high percentage of similarity among human, chicken and egg isolates.

- Upon curing the derivative strains CE2, CE7, CH32,and CH62 became completely sensitive to the all antibiotics tested. The cured derivatives CH32 and CH62 were found to lost all of their plasmids.

- All the cured derivatives lost the ability to express long-chain LPS and became rough.

- The strain CA13, the cured derivative of PT6 A13 isolate, became PT21. The strain CE7, the cured derivative of PT20a E7, became PT20 variant.

- The strain CA13, the cured derivative of OMP-typeIII A13 isolate was found to have conversion to OMP-typeII.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Luria Broth

Component:	g/L
Peptone	10.0
Yeast extract	5.0
NaCl	10.0

pH is adjusted to 7.5 and sterilized at 121°C for 15 min.

2. Nutrient Agar

Component:	g/L
Agar	15.0
Nutrient Broth	13.0

Sterilized at 121°C for 15 min.

APPENDIX B

BUFFERS

1. TE Buffer

Tris 0.3 g

EDTA 0.08 g

Complete volume to 250 mL and adjust pH to 8.0

2. Lysis Solution

0.1 N NaOH

1% SDS

3. Potassium Acetate

Potassium acetate 14.721 g

Complete volume to 40 mL by adding distilled water and then adjust pH 4.8 to 5.0.

4. Agarose Gel Staining Solution

1.0 µg/mL ethidium bromide in distilled water

5. Satacking Buffer

6 g Trizma-base is dissolved in 80 mL distilled water, adjusted pH 6.8 with concentrated HCl and volume is completed to 100 mL.

6. Separating Buffer

Trizma-base	36.6 g
1N HCl	48 mL

diluted to 100 mL by using distelled water

7. Running Buffer

Trizma-base	3 g
Glycine	14.4 g

volume is completed to 500 mL with distilled water and SDS is added freshly to give final concentration of 1 g/L.

8. Sample Buffer (10 mL)

Autoclaved glycerol	2.2 ml
10% SDS	0.1 mL
β-Mercaptoethanol	0.5 mL
Stacking Buffer	1.25 mL
Sterile distilled water	5.95 mL
Bromophenol blue (arbitrary)	

9. Comassie Brillant Blue Solution

Comassie brillant blue	0.1% (w/v)
Glacial Acetic acid	12% (v/v)
Methanol	50% (v/v)

10. Destaining Solution

Methanol	30% (v/v)
Acetic acid	70% (v/v)

11. Fixation Solution (300 ml)

Isopropanol	75 mL
7% Acetic acid	225 mL

12. Oxidation Solution

Periodic acid	1.05 g
50% Acetic acid (v/v)	6 mL
Ethanol	4 mL

Complete volume to 150 mL

13. Silver Staning Solution

Silver nitrate	1 g
0.1N NaOH	28 mL
29.4% Ammonium hydroxide	3 mL

Add 113 mL distilled water.

14. Development Solution

Citric acid	0.05 g
37% Formaldehyde	500 mL

Complete volume to 1 L.

15. Stop Solution

7% Acetic acid (v/v)	10 mL
Distilled water	200 mL

16. 45% Acrylamide GelStock Solution

Acrylamide	112.5 g
Bis-acrylamide	2 g

Complete volume to 250 mL byadding distilled water.

17. Detergent Solution

Laurylsarcosine	16.7 g/L
Tris	1.34 g/L

18.PBS Solution

NaCl	8.47 g/L
NaH_2PO_4	0.3 g/L
Na_2HPO_4	1.065 g/L

APPENDIX C

CHEMICALS AND SUPPLIERS

8-Hydrogen Quinoline..	Sigma
Acetic Acid..	Merck
Acrylamide...	Sigma
Agar Bacteriological..	Sigma
Agarose...	Sigma
$AgNO_3$..	Sigma
Ammonium persulfate...	Merck
Bisacrylamide...	Sigma
Bromophenolblue...	Merck
Chloroform...	Merck
EDTA..	Sigma
Ethanol..	TEKEL
Formaldehyde...	Sigma
Glycerol...	Merck
Glycine..	Sigma
Isoamyl alcohol..	Sigma
Mercaptoethanol...	Merck
Methanol..	Merck
Phenol..	Sigma

Potassium acetate..	Sigma
RNAse...	Sigma
SDS..	Merck
TEMED..	Sigma
Antibiotic discs...	Sigma
CTAB...	Sigma
Sodium-laurylsarcosine..	Sigma
Sodium citrate..	Merck
Proteinase K...	Sigma
NaH_2PO_4..	Sigma
Na_2HPO_4..	Sigma
Yeast extract..	Sigma
Tryptone..	Sigma
Periodic acid..	Sigma
Citric acid..	Sigma
Ammonium hydroxide..	Merck
Methylene bis-acrylamide...	Sigma
Urea..	Merck

VITA

Bülent İÇGEN was born in Ankara on April 13, 1968. He finished primary and high schools in the same place. Then he received his B.Sc. degree in Biology from Hacettepe University in 1990, and his M.Sc. degree in Biotechnology from Middle East Technical University in 1994. He continued in the same department for the degree of Doctor in Philosophy at Middle East Technical University. Fluorescein *In Situ* Hybridization (FISH) Techniques, Electrophoretic Techniques (SDS-PAGE, PAGE and Agarose), Spectroscopic Techniques, Microbiological Tests and Molecular Biology Techniques are among the practical techniques that he knows well. He is married with a son and fluent in English and German.