MOLECULAR DETECTION OF QUINOLONE RESISTANCE AMONG NON-TYPHOID *SALMONELLA* FROM CLINICAL AND FOOD ISOLATES

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MOLECULAR DETECTION OF QUINOLONE RESISTANCE AMONG NON-TYPHOID *SALMONELLA* FROM CLINICAL AND FOOD ISOLATES

الكشف الجيني عن السالمونيلا الغير التيفوئيديه المقاومه لل **Quinolone** في العينات السريريه و الطعام

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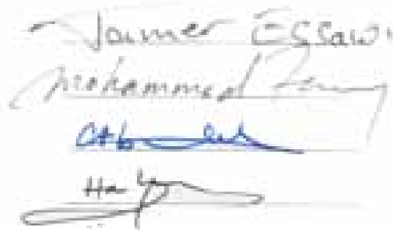

Dedication

I dedicate this thesis to my dear father and mother, Rabah and Hane and my brothers Rami, Fouad, Ibrahim and Samer

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RRD

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List of Abbreviations

Ala	Alanine
AMR	Surveillance for Antimicrobial Resistance
Asn	Asparagine
Asp	Aspartic acid
CDC	Center for Disease Control
CLSI	Clinical Laboratory Standard Institute
CRT	Prolonged Capillary Refill Time
Cys	Cysteine
D	Aspartic Acid
F	Phenylalanine
FDA	Food and Drug Administration
Gly	Glycine
NTA	Non-Typhoid *Salmonella*
Phe	Phenylalanine
QRDR	Quinolone Resistance Determining Region
S	Serine
SCV	Salmonella Containing Vesicle
Ser	Serine
SPI	*Salmonella* Pathogenicity Islands
TAE	Tris-EDTA buffer
Tyr	Tyrosine
XLD	Xylose-Lysine-Dextrose

Abstract

Salmonella are usually transmitted to human by the consumption of contaminated foods and water. The prevalence of *Salmonella* varies depending on the water supply, waste disposal, food preparation practices, and climate. The highest incidence rates occur in children younger than 5 years of age, particularly those younger than 1 year, and in individuals older than 70 years of age.

Human Salmonellosis caused by non-typhoidal *Salmonella* species (NTS) occurs with increasing frequencies in developed countries. The emergence of fluroquinolone resistant *Salmonella* poses a serious problem. Fluroquinolones are considered the treatment of choice in cases of acute salmonellosis. The increase in antimicrobial resistance has lead many countries to initiate surveillance program to monitor dissemination and detect the evolution of bacterial resistance.

In this study, we evaluated the antimicrobial susceptibility of (NTS) following the Clinical Laboratory Standard Institute recommendations. In addition, we determined the molecular mutations on *gyrA* that caused resistance to quinolones by PCR. To confirm and specify the changes that occurred on *gyrA,* representative samples were sequenced.

Our results revealed that *Salmonella* serogroups C and D were the most common among clinical isolates, while serogroups C and B were the most common among food isolates. Antimicrobial susceptibility testing revealed that all strains tested were susceptible to ceftriaxone, most of the strains were susceptible to chloramphenicol, gentamicin and trimethoprim sulfametaxazole. To evaluate resistance to quinolones, nalidixic acid and ciprofloxacin were used. NTS were divided into three main groups designated as: Susceptible to both quinolones (SS), resistant to nalidixic acid but susceptible to

ciprofloxacin (RS) and resistant to both (RR). Antimicrobial susceptibilities in the 3 groups in clinical and food isolates were 38% and 51% (SS), 30% and 30% (RS) and 30% and 15% (RR) respectively.

The *gyrA* gene was amplified and resolved by agarose gel electrophoresis. A sharp band of 630 bp was obtained and restricted by *Hin*f I enzyme. Subsequent agarose gel electrophoresis revealed various size bands depending on the pattern of susceptibility or resistance of *Salmonella* isolates to nalidixic acid and ciprofloxacin. The SS pattern revealed 3 major bands of 250 bp, 150 bp and 100 bp. A faintly appearing fourth band of about 130 bp was also seen. Identical results were obtained with the RS group, with the RR pattern; two major bands of 350 bp and 150 bp were evident. A faintly appearing third band of 130 bp was also seen.

The sequence results obtained for the SS pattern was normal with no mutations. The sequence obtained with the RS pattern revealed a mutation at position 87. A substitution of G to A (GAC became TAC). The sequence obtained with the RR pattern revealed two mutations at positions 83 and 87. At position 83, a substitution of C to T (TCC became TTC) was detected. At position 87, the same type of mutation occurred as in the RS pattern was detected, a substitution of G to A (GAC became TAC).

The level of resistance to quinolones in this study among NTS isolates is alarming. This warrants the prohibition of the use of quinolones in chicken feed and the restriction of administring of quinolones without a prescription.

ملخص

السالمونيلا. تصل إلى الإنسان عن طريق الغذاء أو الطعام أو الماء الملوث، ونسبة الانتشار تعتمد على مدى سلامة نظام شبكة المياه، وسلامة تصريفها، إلى جانب العوامل البيئية مثل المناخ والطقس السائد في تلك المنطقة. النسبة الأعلى من الإصابة بهذه البكتيريا هو في الأطفال الذين تقل أعمارهم عن خمس سنوات وبالأخص تحت عمر السنة، كذلك البالغين فوق عمر السبعين.

تسبب السالمونيلا الغير التيفوئيدية التسمم الغذائي عند الإنسان عند التعرض لها وهذا شائع في الدول النامية. إن السالمونيلا المقاومة لمجموعة "Fluoroquinolone" تشكل خطراً كبيراً حيث أن هذه المجموعة من العلاج هي العلاج الأساسي للصنف المقاوم للمضادات الحيوية الأخرى، مما حدا بكثير من الدول لعمل برامج إنقاذ ومن الحد من انتشار والسيطرة من هذا النوع من البكتيريا.

الدراسة التي أمامكم قمنا بتقييم مدى فعالية بعض أنواع الأدوية اعتماداً على توصيات "CLSI".وحددنا الطفرة في ألجين المسمى "*gyrA* " المسؤول عن جعل البكتيريا مقاومة لمجموعة Quinoloune وذلك عن طريق PCR ولتحديد ما يحصل من تغييرات في ألجين *gyrA* قمنا بتحضير مجموعة من العينات لتحليلها، قسم منها مأخوذ من الإنسان والقسم الآخر مأخوذ من الغذاء ووجدنا التالي:

العينات المأخوذة من الإنسان كانت تحتوي على المجموعة C,D بينما العينات المأخوذة من الغذاء كانت تحتوي على C,B ، كما وجدنا أن كل العينات حساسة للدواء السفتريكسون، ومعظمها حساسة لمجموعة من الأدوية الأخرى مثل كلورمفنيكول، الجنتاميسين، ترايميثوبريم..

لمعرفة مدى المقاومة لمجموعة Quinoloune استخدمنا نلاديكسيك اسيد والسبرواوفلاكسسين. البكتيريا الغير المسببة للتيفوئيد قسمت إلى 3 مجموعات: مجموعة حساسة لصنفي الQuinoloune، مجموعة حساسة لصنف واحد منهما، مجموعة مقاومة للصنفين.نتائج فحص حساسية البكتيريا الغير مسببه للتيفوئيد في المجموعات الثلاث من الطعم و من العينات السر يريه كانت كما يلي بالترتيب:38 % و 51 % (SS), 30% , 30% (RS) و30%, 15% (RR)

تم عزل ألجين *gyrA* باستخدام وتحليل جل الأغار على النقطة 630 bp ، التي تم عزلها وتقطيعها بواسطة الإنزيم *Hinf* I. لوحظ عدّة شرائح "قطع" مختلفة الأحجام اعتماداً على حساسيتها أو مقاومتها لصنفي الدواء. من تحليل النتائج وجدنا أن البكتيريا الحساسة لصنفي الدواء كانت لا تحتوي على طرفة جينية، بينما البكتيريا المقاومة لنوع واحد من الدواء كانت تحتوي على طفرة جينية واحدة على الموقع 87 والمجموعة المقاومة لصنفي الدواء تحتوي على طفرتين جينيتين على الموقع 83و 87.

نستنتج من نتائج هذه الدراسة أن نعمل جاهدين للحد من استخدام دواء Quinoloune في أطعمة الدواجن والحيوانات بدون وصفة الطبيب.

Introduction and Review of the Literature

1.1 General characteristics of Salmonella*e spp*.

Salmonellae are gram-negative bacilli, motile, non-encapsulated, non-spore-forming facultative anaerobes. It is named after the American microbiologist, Daniel Elmer Salmon. Most strains ferment glucose, maltose, and mannitol but do not utilize lactose or sucrose. All pathogenic *Salmonella* other than *S. typhi* produce gas. A wide variety of selective differential agars have been developed for the isolation of *Salmonella* from clinical specimen. All these agar based media contain sugars, dyes and bile salts (Cassar, and Cuschieri, 2003). Differentiation of most salmonellae relies on the results of biochemical tests such as the production of hydrogen sulfide and the non-fermentation of lactose. The most commonly used media include *salmonella-shigella* agar, Hektoen enteric agar, brilliant green agar, and Xylose-lysine-dextrose agar (XLD). Enrichment in selenite broth usually results in the inhibition of normal fecal flora such as Coliforms and enhancing the growth of *Salmonella*.

Salmonellae live in the intestinal tracts of humans and animals. *Salmonella typhi* and *paratyphi* A are strictly human pathogens. The transmission of *Salmonella* occurs through fecal contamination of food and water. Although *Salmonella* doesn't multiply well in the natural environment, but under favorable conditions they can survive for weeks in water and several years in the soil.

1.2 Classification

The genus *Salmonella* is a member of the family Enterobacteriaceae. The taxonomy of the salmonellae is the most complex of all Enterobacteriaceae. Classification of *Salmonella spp*. into serovars was done in accordance with the Kauffmann–White scheme, which gives serovar status to each antigenic type on the basis of somatic (O), capsular (Vi) and the flagellar (H) antigens.

There are two species in the genus *Salmonella*, *Salmonella enterica* and *Salmonella bongori,* formerly known as subspecies V *Salmonella enterica* is composed of six subspecies: subspecies *enterica I*, subspecies *salamae II*, subspecies *arizonae IIIa*, subspecies *diarizonae IIIb*, subspecies *houtenae IV*, and subspecies *indica VI,* (Murray, and Baron 1999; Anderson and Ziprin, 2001). *Salmonella enterica* subsp. *enterica*, subspecies I strains are commonly recovered from humans and warm blooded animals. Subsp. II, IIIa, IIIb, IV, VI strains and *S. bongori* are commonly isolated from environmental sources and cold blooded animals ((Murray, and Baron 1999). For the sake of simplicity, the CDC recommends that *Salmonella* species be referred to only by their genus and serovar such as *Salmonella typhi* instead of the *Salmonella enterica* subspecies *enterica* serovar *typhi.*

There are more than 2,435 serotype *Salmonella*, most of which including *Salmonella typhi* belong to subspecies I. Serotyping of *Salmonella* is useful for diagnostic purposes and an important epidemiologic tool for defining out-breaks. *Salmonella spp.* are serotyped according to the "O" heat-stable somatic or cell wall antigens, the "H" flagellar antigens and the "Vi" virulence or capsular antigens. The Vi antigens are heat labile-capsular antigens found only on *S. typhi, S. paratyphi* C and *S. dublin*. The

capsular antigens may interfere and mask the O antigen so the bacteria will not agglutinate with the O antibodies.

1.3 *Salmonella* Pathogenesis

The primary mode of transmission of *Salmonella* is the fecal-oral route; however, airborne transmission is also possible. Oliveira *et al* found that pigs could be experimentally infected by inhaling Salmonella over short distances (Oliveira, et al 2006).

Both non-specific and specific host defenses attempt to suppress infection with pathogenic organisms. After ingestion, the organism must survive the acid mileau of the gastric fluid. The vast majority of the bacteria perish in the stomach, but with a large inoculum, enough bacteria survive to reach the distal small intestine and colon. A study in mice, suggests that gut luminal contents and composition may be critical for establishing infection in the intestinal epithelium (Clark, *et al*, 1998). *Salmonella typhimurium* failed to infect mice intestinal cells in vitro when inoculated in phosphate buffered saline (PBS), but invasion was enhanced with Luria-Bertani (LB) broth. The authors speculate that amino acid supply may be an essential signal for cellular invasion, since addition of tryptone and yeast extract to PBS (two ingredients of LB broth), resulted in epithelial infection by *Salmonella*. This suggests that salmonellae receive environmental cues that change their extra cellular lifestyle and invade target intestinal epithelial cells (Meyerholz, 2002).

Bacteria preferentially adhered to microfold (M) cells associated with the follicle-associated epithelium (FAE) initially, but later were observed invading other cell

types as well (goblet cells and enterocytes). This study suggests that M cells are targeted early in disease since they lack surface barriers such as glycocalyx and mucus, which is present on absorptive enterocytes and goblet cells (Van Asten, *et al* 2005) Bacteria were also observed migrating through crevices formed by extruded enterocytes (a process of normal cell turn over), suggesting that *Salmonella* takes advantage of these sites for invasion as well (Figure1).

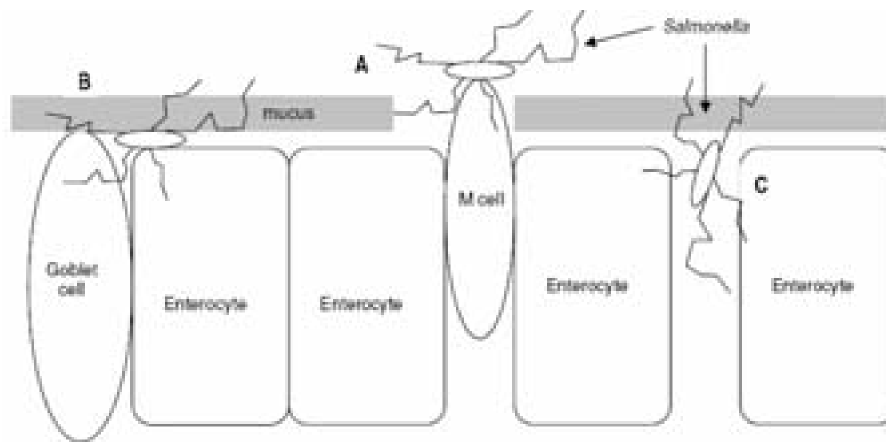

Figure1: Various mechanisms for *Salmonella* invasion. Early in cellular invasion, *Salmonella* adheres and preferentially targets epithelial M cells (A) which lack surface barrier of mucus and glycocalyx. In later stages of infection, *Salmonella* can be observed invading enterocytes and goblet cells (B) or migrating through crevices formed by extruded enterocytes (Van Asten, *et al* 2005)

Bacteria have evolved unique mechanisms for invading non-phagocytic host cells. This "forced entry" into cells allows pathogens to evade host defenses and invade target tissues. There are a multitude of gene products that are required for full virulence and host interaction. In enteric bacteria, these genes are arranged into large clusters on the chromosome called pathogenicity islands (SPI) so the proteins they encode can be efficiently produced at the correct time and location. *Salmonella* spp. possesses five pathogenicity islands, each encoding different virulence factors that are needed for different phases of bacterial invasion.

Further plasma membrane manipulations create invaginations and eventually seal off around the invading microorganism. This is often termed as macropinocytosis since it resembles the normal cellular mechanism of pinocytosis, but on a much larger scale. Once internalized, *Salmonella* have evolved mechanisms for avoiding degradation by lysosomal vesicles. The *Salmonella* containing vesicle (SCV) continues to manipulate the host cell by recruiting actin molecules to the vacuole surface. This actin coat may act as a protective barrier preventing fusion with host oxidase-containing vacuoles. Secluded in the SCV, the pathogen can replicate by harnessing components of the host cell. Translocation of bacteria to adjacent cells can occur across the basolateral border trafficking them into deeper structures of the lamina propria. In this region, the bacteria gain access to phagocytic cells, especially tissue macrophages for invasion and eventual dissemination to other organs.

One study demonstrated that the spread of *Salmonella* from the intestinal mucosa to the liver and spleen is dependent on CD18-expressing macrophages and possibly dendritic cells (Vasques-Torres, *et al*., 1999). Infected phagocytic cells may allow cell mediated immune mechanisms to contain the infection. In neonates or the immunocompromised host, extraintestinal infections can occur resulting in septicemia, pneumonia, septic arthritis or meningitis. Some speculate that infected phagocytes may play a permissive role in allowing state of latent persistent infection (McCain, *et al* 1990; Powell, 1994). Studies in mice have found *Salmonella typhimurium* bacteremia peaked 30 minutes after gastrointestinal inoculation (Vasques-Torres, *et al*., 1999), and *Salmonella* can be identified in the ileocecal lymph nodes in esophagotomized pigs within 6 hours of intranasal inoculation (Fedorka-Cray, 1995).

1.4 Mechanisms of Diarrhea

The classic feature of clinical salmonellosis in most vertebrate species is profuse, voluminous diarrhea. This occurs as a result of intestinal fluid losses by two mechanisms: (1) active fluid loss through secretory hyperstimulation and (2) passive fluid loss by inflammation mediated malabsorption. *Salmonella* produces various virulence factors including exotoxin, cytotoxin, enterotoxin and endotoxin that mediate the development of diarrhea (Murray,1986). Cytotoxin, as the name implies, causes intestinal epithelial cell damage either directly through chelation of cations in the mucosal cell membrane or indirectly via stimulation of cytokines and inflammation. By inflicting direct damage of absorptive villous enterocytes, bacterial cytotoxins lead to reduced absorptive capacity and result in loss of electrolytes and water. Another mediator of inflammation is bacterial LPS or endotoxin, which through its interaction with local macrophages triggers a profound inflammatory effect resulting in the influx of neutrophils.

Inflammatory mediators released by infiltrating leukocytes, such as cytokines, enzymes and oxygen species, provoke local tissue damage and contribute to the breach in mucosal integrity and intestinal malabsorption. Bacterial enterotoxins initiate diarrhea by binding to receptors that stimulate the second messenger systems of cAMP and cGMP, which secondarily activate enterocyte fluid hypersecretion (Jones, and Blikslager, 2002).

Clinical salmonellosis is characterized by explosive and voluminous diarrhea, abdominal discomfort and systemic signs of toxemia including fever, mucous membrane injection with prolonged capillary refill time (CRT), anorexia, and

depression. Accompanying signs of circulatory shock may be present especially if infection has advanced to bacteremia, including tachycardia, cool extremities, poor pulse quality and weakness. Milder infections are usually self-limiting, and patients may improve clinically in a relatively brief period of time. An important clinical feature of salmonellosis is the potential for some infected animals to shed the organism without demonstrating clinical signs of the disease (silent shedder). These inapparent carriers are capable of introducing the organism (although often in low numbers) into the environment to other susceptible hosts (Smith, 1979).

1.5 Factors Influencing Infection

There are a variety of factors that will influence the development of clinical salmonellosis. Some of these factors are related to the microorganism itself, such as the infective dose of bacteria and the virulence of individual strain. Other factors that influence infectivity are related to the host and its individual susceptibility. Studies in mice have demonstrated that the number of *Salmonella enteritidis* organisms required to infect conventionally raised animals is 10^6 or greater. However, in germ-free mice, only 10 bacteria were necessary to result in diarrhea, septicemia and death (Collins, 1978).

Salmonella virulence is also dictated by a number of cellular components of the bacterium itself. These include adhesion molecules, toxins (cytotoxin, endotoxin, enterotoxin) and antimicrobial resistance. Some virulence factors are encoded in the microbial chromosome and some are located on extrachromasomal plasmids.

Bacteria can acquire new virulence factors or modify their existing ones by two methods: spontaneous genetic mutations and transfer of DNA among bacteria.

Spontaneous mutation are not common, however, because bacteria have a short generational interval, mutations can be propagated exponentially. Fortunately, spontaneous mutations do not impact virulence factors and antimicrobial resistance patterns to a large degree (Dargatz and Traub-Dargatz, 2004). Unfortunately, bacteria can also share virulence factors by horizontal gene transfer with other bacteria. Bacterial conjugation is the transfer of genes by cell to cell contact usually by means of pilli. Bacterial transformation is the ability of bacteria to acquire environmental DNA across the cell membrane and integrate it into its genome. Transduction is the method for acquiring genes through vectors such as bacteriophages. Through these methods, bacteria can effectively acquire resistant genes not only from *Salmonella,* but from different bacterial genera as well in recent decades, the emergence of highly-virulent, multidrug-resistant *Salmonella* strains has become of great concern.

1.6 *Salmonella* in Food

Serovars of *Salmonella enterica* are the primary bacterial agents responsible for foodborne outbreaks of human gastrointestinal diseases. Among the different species, *S. enteritidis* and *S. typhimurium* serovars are of particular clinical importance. *Salmonella* spp. especially *S. enterica* serovars are pathogenic bacteria which frequently contaminate food products particularly those containing poultry, eggs, meat, dairy products, fruits and vegetables contaminated with animal feces or handling contaminated pet treats (Bearson, and Shawn*,* 2006; Poppe, *et al.,* 2006; D'Aoust, 1991; Nguyen and Khan **,** 1994).

In 1958, an outbreak of salmonella caused by foodborne *S. enterica* serotype *hadar* in Israel was linked to consumption of chicken liver. An investigation of the chicken

farm found that bone meal fed to the chickens was contaminated with the same serotype of *Salmonella* (Hirsch, *and* Sapiro-Hirsch*.*, 1958). Milk borne outbreak due to *S. enterica* serotype *heidelberg* in England in 1963 was traced to a cow with bovine mastitis due to the same organism. Chickens are usually infected by oral uptake of bacteria from the environment. Contaminated litter is the most important source of infection. Besides this horizontal transmission, vertical transmission through eggs is also considered as a source of infection in chickens (Poppe, 2000). It is known that *S. enteritidis* can persist in chicken organs and intestine and predominantly colonizes the carcass of the animal (Desmidt, *et al*., 1998a, 1998b). Between 1988 and 1992, averages of 110 outbreaks of *Salmonella* were reported each year to CDC (Bean *et al*., 1996). Sixty percent of these outbreaks were caused by *Salmonella* serotype *enteritidis* and most of these were attributed to eating undercooked eggs. Many of these egg-associated outbreaks were traced back to their farm of origin and it was demonstrated that infected hens were the source of the outbreak.

Outbreaks of *Salmonella* infections associated with eating Roma tomatoes were detected in the United States and Canada in the summer of 2004. An outbreak was characterized by a single *Salmonella* serotype. There were 561 cases in an outbreak-related illness from 18 states and one province in Canada. Although a single tomato-packing house in Florida was common to all three outbreaks, other growers or packers also might have supplied contaminated Roma tomatoes that resulted in some of the illnesses. Because current knowledge of mechanisms of tomato contamination and methods of eradication of *Salmonella* in fruit is inadequate to ensure product safety, further research should be a priority for the agricultural industry, food safety agencies, and the public health community (CDC, 2005).

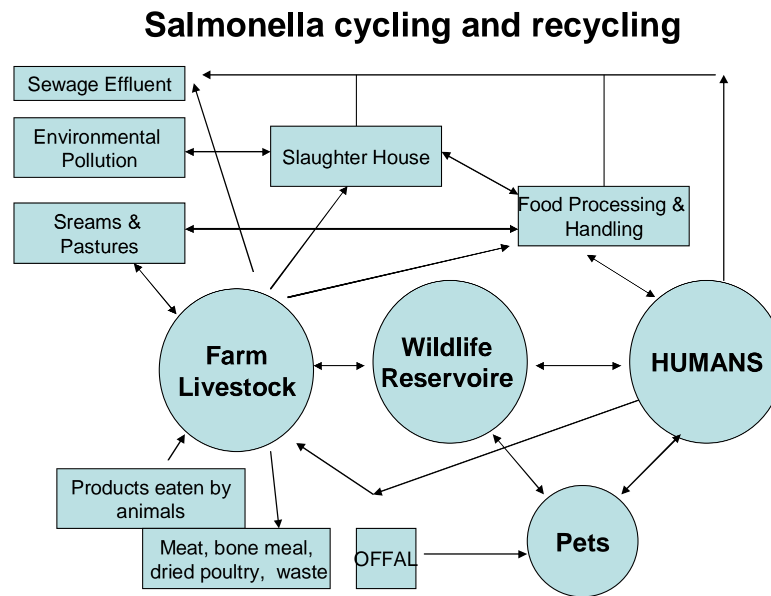

Figure2: Schematic representation of the cycling of NTS between animals, food and humans.

An increase in antibiotic resistance was observed due to the emergence of an epidemic multidrug-resistant (MDR) strain of serotype Typhimurium of definitive phage type 104 (DT104), especially in Europe and North America (Weill, et *al*., 2006; Threlfall, 2000). In these DT104 isolates, multiple antibiotic resistances were due to chromosomal integration of a 43-kb structure called *Salmonella* genomic island 1 (SGI1). Foods of animal origin are frequently implicated in human salmonellosis owing to the high prevalence of *Salmonella* strains in animals (Oosterom, 1991). The most frequent causes of infection by *Salmonella* reported in humans have been through the handling of raw poultry carcasses and products, together with the consumption of undercooked poultry meat (Panisello, *et al.,* 2000).

The number of reported cases of salmonellosis has increased about threefold in the United States during the past 20 years (Tauxe, 1997). In the past, foodborne outbreaks were often local occurrences, affecting people who attended the same social event or who ate at the same restaurant (Slutsker, *et al.* 1998). Today, evidence suggests that outbreaks are increasingly cross provincial, territorial and national borders (Tauxe, and Hughes, 1996). Salmonellosis is responsible for heavy economic losses in both developing and developed countries to the commercial poultry industry through morbidity, mortality and reduced egg production (Pomeroy and Nagaraja, 1991).

Among the more than 2,500 serotypes of the genus *Salmonella* described to date (Popoff, 2001), *S. enteritidis* and *S. typhimurium*, are predominant in many developed countries. *S. enterica* serotype *typhimurium* was the second most prevalent serotype (*S. enteritidis* ranked first) in Europe during the period 1998 to 2003 (Fisher, 2004). It has a large animal reservoir, including farm animals, pets, and wild animals. Although most *Salmonella* infections cause mild diseases (gastroenteritis), life-threatening infections (e.g., bacteremia) may occur, particularly in cases involving patients at the extremes of age or those who are immunocompromised.

Acquisition of an incompatibility group HI plasmid can encode simultaneously resistance to chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracyclines (Couturier, *et al.,* 1988; Fica, *et al*., 1997; Rowe, *et al*., 1997). Quinolone resistance, which is associated with chromosomal point mutations in the *gyrA* gene, has been also reported (Wain, *et al.,* 1997). Studies showed that high rate of bacterial resistance to antibiotics in animals is likely due, in part, to selective pressure resulting from misuse of these antibiotics, improper treatment, prophylaxis or commonly given

antimicrobial growth promoters (Barza, 2002; Pedersen, 1999; Witte, 2000; Van den Bogaard and Stobberingh, ,2000).

Eradication of *Salmonella* isolates from the surrounding environment is almost unattainable. Therefore, preventive control measures must be adopted. Guidelines must be adopted to control contaminated sources such as primary animal production, slaughterhouses, and food-producing plants, careful handling of raw products and end products to limit the recurrence of disease. Therefore, poultry industry has to routinely apply detection techniques for *Salmonella* to assess contamination risk in their production chain. It is also desirable that the monitoring methods be applied to large numbers of samples at low costs. Early, diagnosis, laboratory-based surveillance including *Salmonella* serotyping and monitoring of resistance, immunization, and public education, is essential for prevention and control (Rijpens and Herman, 2002; Slutsker *et al.,*. 1998). Efforts for control of *Salmonella* are under way at the local, national and international levels to improve the capacity, coordination and cooperation of public and private laboratories, physicians, hospitals and public health officials (Slutsker, *et al.,* 1998, Tauxe and Hughes, 1996).

1.7 Epidemiology

Non-typhoidal salmonellosis is a foodborne disease of primary concern in developed as well as developing countries. The spread of this disease is favored by wide array of animal reservoir and by the wide commercial distribution of both animals and food products. It is one of the major public health problems in terms of socio-economic impact (Gracia and Finlay,1994) Global surveillance data indicates that incidence of gastrointestinal infections caused by *S. enteritidis* has increased massively during the last decades.

Food contamination with antibiotic-resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance. The prevalence of antimicrobial resistance among food-borne pathogens has increased during recent decades (Boonmar, *et al.,* 1998; Chiu, *et al*., 2002; Davis, *et al*., 1999; Threlfall*,* 2000), possibly as the result of selection pressure created by the use of antimicrobials in food-producing animals (Threlfall, *et al.,* 1997; Angulo, *et al*., 2000; Bywater, 2004; Teuber, 2001). The coexistence of resistance genes with mobile elements such as plasmids, transposons, and integrons facilitates the rapid spread of antibiotic resistance genes among bacteria (Sunde**,** *et al*., 2006). Molecular analysis of antibiotic resistance genes and antibiotic-resistant mobile elements has shown that identical elements were found in bacteria that colonize both animals and humans, suggesting a role for raw foods in the dissemination of resistant bacteria and transfer of resistance genes to humans via the food chain (Levy*, et al*., 1976; O'Brien, *et al* 1984; Teuber 2001).

An increase of multidrug resistant *Salmonella* strains showing resistance against different antibiotics have been recovered from isolates of humans, poultry, and cattle origin in recent year (Esaki, *et al.,* 2004).

Salmonella typhimurium DT104, resistant to streptomycin, chloramphenicol, amoxicillin, sulfonamides, and tetracyclines, is one of the strains that most frequently displays multiple resistance characteristics (Kristiansen, *et al.,* 2003), additional resistance to quinolones, such as nalidixic acid and ciprofloxacin, has been described in recent years, thus increasing the importance in the public health area (Piddock, and Ricci 1998)**.**

Quinolones are widely used for treatment and prevention in animal production systems, particularly in the aviculture area. The Federal and Drug Administration (FDA), in docket N8 00N-1751, have prohibited the use of enrofloxacin in poultry, due to the increased resistance displayed by *Campylobacter* spp. (FDA, 2000). The antibiotic effect of quinolones depends on its ability to bind to the DNA gyrase complex inducing a conformational change in the enzyme (Hawkey, 2003). In *Salmonella* spp., as well as in *E. coli* and in the majority of gram-negative microorganisms, resistance to quinolones is determined fundamentally by two mechanisms: one corresponds to the alteration of the DNA gyrase binding site due to mutation between amino acids 67 and 106 of the QRDR region (Quinolone Resistance Determining Region) of the *gyrA* gene. The other mechanism corresponds to structural changes of the porins of the outer membrane of gram-negative microorganisms, which produce an alteration of the permeability of the bacteria to the antimicrobial agent (Reyna, *et al.,* 1995).

The most frequent mutation in *gyrA* gene which confer resistance to nalidixic acid, are those located at the Ser 83 or Asp 87 amino acids. The mutation in Ser 83 can change the amino acid to Phe, Tyr, or Ala, while the mutation in Asp 87 can change the amino acid for Gly, Asn, or Tyr. Double mutations for both amino acids confer resistance to ciprofloxacin, as observed in isolates of *Salmonella* spp. (Reyna, *et al.*, 1995). A less frequent mutation associated to resistance is found on amino acid Gly 81, which is substituted for Asp or Cys (Giraud, *et al.,* 1999).

Information on the phenotypes and genotypes of antimicrobial resistance in foodborne microorganisms is largely restricted to developed countries, and there is a paucity of information on what is happening in developing countries. Where they are reported, rates of resistance to antibiotics of bacteria originating from meat were high in developing countries (Al-Ghamdi, *et al.,* 1999; Angkititrakul, *et al.,* 2005; Chung, *et al.,* 2003; Manie, *et al.,* 1998; Taremi, *et al.,* 2006), possibly as the result of the inappropriate or uncontrolled use of antibiotics in farming practices. Therefore, the study of antibiotic resistance in developing countries is important as the information could enhance prudent use of antibiotics in food production. In Palestine, antibiotic resistance has not been reported. However, as far as we are aware, there have been no published reports about the occurrence of antibiotic-resistant bacteria in raw food samples in Palestine and even no reports about the molecular characteristics of these antibiotic-resistant bacteria.

Several studies about salmonellosis have been recently done on children in Israeli hospitals. *Salmonella* was found in 25% of all stool cultures examined as compared to 30% *Shigella* and 2% *Campylobacter* (Finkelstein, *et al.*, 2002). Another study

conducted from 1995 to 2003, had reported that the incidence of endemic fever was 2.7 times higher in Arabs than Jews (Meltzer, and Yessepowitch, 2006). Investigations of diarrheal outbreaks among Israeli soldiers between 1998 and 2003 revealed that *Shigella*, *Salmonella* and *Staphylococcu*s spp. were the most commonly isolated organisms (Schwaber, *et al.,* 2005). In a Jordanian study performed on 400 patients with diarrhea, *Salmonella* was isolated in 2.3% of the cases (Shehabi *et al*, 2001). In a case study performed on the swimming pools in the West Bank, *Salmonella* was the most commonly isolated organism (Al-Khatib and Salah., 2003). A study on the epidemiology of *Salmonella* and *Shigella* conducted from 2001 and 2005 revealed that the majority of *Salmonella* spp. has affected children less than two years of age. *Salmonella* O group C, serovars *virchow*, *hadar, newport* and *kentucky,* and *Salmonella* O group B server *typhimurium* were the most common (Ghneim, *et al* 2006).

1.8 Therapy

Acute salmonellosis is largely supportive. Aggressive intravenous fluids, treatments aimed at ameliorating the effects of endotoxemia (nonsteroidal anti-inflammatories, plasma, antisera, polymyxin B), and intestinal protectants are the mainstay of therapy. Probiotic therapy with commercial *Lactobacillus* preparations has shown from time to time benefits in salmonellosis through the production of inhibitory factors and competition for mucosal colonization. Antibiotic therapy in patients with *Salmonella* colitis remains a controversial area. Antibiotics are not indicated unless the patients are at high risk of developing septicemia, such as in the case of neonates or other immunocompromised patients. Fluoroquinolones are broad-spectrum antimicrobials effective in the treatment of a wide variety of clinical and veterinary infections and are the antimicrobials of choice for treatment of invasive gastrointestinal infections in adults, in many parts of the world. *Salmonella* gastroenteritis is usually treated with fluoroquinolones when the patient is elderly or immunocompromised (Hopkins, *et al.,* 2005). Many suspect that antibiotics may in fact prolong the period of bacterial shedding and there is increasing evidence that inappropriate antibiotic use may contribute to the emergence of drug resistant salmonellae through bacterial selection pressures.

1.9 Quinolones

1.9.1 Quinolones Structure

Quinolones are synthetic antimicrobial agents belong to a group of potent antibiotics. Their structure contains two fused rings, with a carboxylic acid at carbon-3 and a ketone at carbon-4. Nalidixic acid, the first generation quinolone and the only one approved for use in children, is a non-fluorinated compound synthesized in 1962 and still in use today (Figure 3). Modification of the nalidixic acid gave rise to the broad spectrum and highly effective drugs known as fluoroquinolones developed at the beginning of 1980s (e.g perfloxacin, ciprofloxacin, and ofloxacin) as shown in Figure 4. This modification has a much greater activity against bacteria such as Enterobacteriaceae, *Staphylococci, Pseudomonas aeruginosa, Mycoplasma* and *Chlamydia.* The fluoroquinolones were synthesized by adding a fluorine atom at carbon-6 of the parent nalidixic acid compound (Leibovitz, 2006).

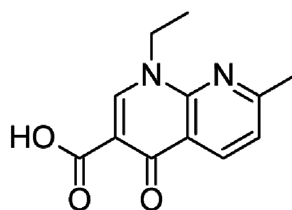

Figure 3: The chemical structure of the parent quinolone, nalidixic acid.

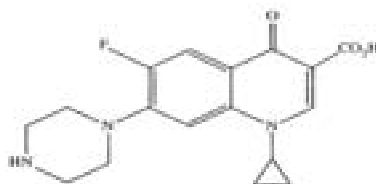

Figure 4: The chemical structure of ciprofloxacin, a fluoroquinolone

1.9.2 Mechanism of Action and Spectrum of Activity

Bacterial topoisomerases are a class of enzymes essential in maintaing the bacterial DNA molecule stable and biologically active including DNA gyrase, a type II DNA topoisomerase enzymes and topoisomerase IV (Leibovitz, 2006). DNA gyrase introduces supercoils into the linear DNA double helix, which results in the highly condensed 3-dimensional structure of the DNA usually present inside the cell. During the supercoiling process; both DNA strands are cleaved by DNA gyrase at 4 base pair staggered sites, forming a "quinolone binding pocket". Two quinolone molecules self-assemble inside the pocket in dimer structure and attach to the gyrase-DNA complex electrostatically, which stabilizes the intermediate stage of this reaction step. Permanent gaps in the DNA strands induce synthesis of repair enzymes (exonucleases), initiating uncoordinated repair processes, which results in irreversible damage to the DNA and, finally, cell death. Topoisomerase IV found that in vitro, is to decatenate the daughter chromosomes in the final stages of DNA replication.

DNA gyrase consists of two proteins (A and B), with the active species being a heterotetramer (A2B2). Topoisomerase IV. Comprises two subunits, *ParC* and *ParE.* The *ParC* protein is homologous to the gyrase A protein, while the *ParE* subunit is homologous to the gyrase B protein.

Fluoroquinolones interfere with bacterial DNA metabolism by the inhibition of two enzymes, Topoisomerase II (DNA gyrase) and Topoisomerase IV. In Gram-negative bacteria DNA gyrase is the primary target, whereas in Gram-positive Topoisomerase IV was recently found to be most affected.

1.9.3 Pharmacology:

Fluoroquinolones are generally well absorbed in the gastrointestinal tract. The newer fluoroquinoloes have improved pharmacokinetic properties compared with the older ones, in terms of longer serum half-life, higher peak levels leading to maximal bacterial killing and large volumes of distribution with subsequent extensive tissue penetration (Murray,and Baron, 1999).

Pharmacokinetic data of fluoroquinoloes in pediatric patients are limited. Most studies have been performed in older children with cystic fibrosis and *Pseudomonas spp*. infections. Treatment of such infections requires higher dosage. It was reported by Capparelli *et al*. that rapid absorption and faster clearance of single-dose of gatifloxacin occurs in infants and children as compared with older children and adult patients (Capparelli *et al* 2005).

Quinolones have good penetration into the lungs, kidney, muscle, bone, intestinal wall, and extravascular body fluids. The concentration achieved in the prostate gland is approximately twice that in the serum. The concentration in the urine reaches 25 to 100 times above peak concentrations in serum. However, the concentration in the CSF is rather low (<1 µgml) in patients with meningitis. The Quinolones have the ability to penetrate well into phagocytes, reaching concentrations in the neutrophils and macrophages that are 14 times higher than that in the serum (Ferigin, and Cherry, 1998).

1.9.4 Quinolone Resistance

There are three main mechanisms of resistance to quinolones. First resistance to quinolones occurs with decreased expression of membrane porins. A second mechanism of resistance is expression of efflux pumps in both gram negative and gram positive organisms and the third is alteration of the target enzymes. Several mutations have been described in both quinolone target proteins that result in reduced binding affinities. It is believed that high-level quinolone resistance is brought about by a series of successive mutations in the target genes (Ruiz, 2003).

DNA replication in bacteria occurs via semiconservative mechanism .The bacterial chromosome is circular and interwined. Unwinding of the parental DNA strands during replication usually results in positive supercoiling that would impede the unwinding process. This problem is overcome by the action of DNA gyrase which is capable of removing the positive DNA supercoils and restoring the unwinding process.

DNA gyrase is a tetramer consisting of two nonidentical subunits, A and B in the form A2B2. The A and B subunits are encoded by the chromosomal genes *gyrA* and *gyrB.* The A subunit which is responsible for the breakage and ligation function is the primary target of Quinolones. It has been suggested that quinolones bind to the DNA and DNA gyrase complex causing the DNA strand to be cleaved. Quinolones can then inhibit ligation of the cleaved DNA, thus inhibiting the gyrase function by trapping the covalent enzyme-DNA intermediate (Murray, and Baron, 1999).

Topoisomerase IV is a tetramer in the form A2B2. It is encoded by the *parC* and *parE* genes. Topoisomerase IV is a decatenating enzyme that resolves interlinked daughter chromosomes following DNA replication. Both, DNA gyrase and topoisomerase IV are necessary for cell growth and division. Mutations in one or both genes encoding for the subunits of gyrase and topoisomerase IV may confer resistance to the bacteria against the quinolones.

Among gram-negative pathogens, DNA gyrase is the primary target of the Quinolones. Alterations in the *gyrA* gene are primarily responsible for the development of resistance to this antibiotic. Multiple mutations are probably necessary for expression of high-level fluoroquinolones resistance. The most frequent mutation in quinolone resistant found in *E. coli* at the codon 83 of *gyrA* and in other Enterobacteria, such as *Citrobobacter freundii*, *Shigella spp*, *Neisseria gonorrhoeae* , *Acinetobacter baumannii* and *S. typhimurium* (Ruiz, 2003).

Decreased uptake of these antibiotics is another mechanism for resistance. Decreased quinolones uptake may be associated with two factors: an increase in the bacterial impermeability or the over expression of efflux pumps.

Quinolones may cross the outer membrane in two different ways: through specific porin channels or by diffusion through the phospholipid bilayer. The degree of diffusion of a quinolone is greatly associated with and dependent on its level of hydrophobicity. All quinolones may cross the outer membrane through the porin channels, but only those with the greater level of hydrophobicity may diffuse through the phospholipid bilayer. Thus alterations in the composition of the porins and or in

the lipopolysaccharide-defective mutants, increase susceptibility to hydrophobic quinolones have been described, without alterations in the level of resistance to the hydrophilic quinolones. Alterations in membrane permeability are usually associated with decreased expression of porins. This has been described both in *E. coli* and other gram-negative bacteria (Ruiz, 2003). Recently, the discovery of plasmid-mediated quinolone resistance could result in horizontal transfer of resistance genes between strains (Hopkins, *et al.,* 2005).

Human salmonellosis caused by nontyphoidal *Salmonella* spp. occurs with increasing frequencies in developed countries. The emergence of fluroquinolone –resistant *Salmonella* stains is a serious problem. This class of antibacterial agents is considered to be the treatment of choice in cases of acute salmonellosis due to multi drug resistant strains (Giraud, *et al.,* 1999). The increase in antimicrobial resistance has lead many countries to initiate surveillance program to monitor dissemination and detect the evolution of bacterial resistance (Varavithya, *et al.,* 1990).

It has been proposed that the uncontrolled use of antibiotics in veterinary medicine may promote the emergence of drug resistance bacterial pathogens. This can present a potential risk to public health due to zoonotic infections as seen with *Salmonella* and *Campylobacter*. It has been reported that quinolones are widely administered to farm animals in the United States and Europe. Objections have been raised against these practices in order to minimize the evolution of drug resistance against these antimicrobials (Piddock, and Ricci, 1998; Ruiz, 2003).

This study was conducted to address some of these issues and to provide a current baseline of information on molecular characteristics of antibiotic resistance of *Salmonella* isolates from clinical specimens and foods commonly sold in the marketplace in Palestine. The isolates were investigated to determinate whether quinolone resistant *Salmonella* spp. From human as well as food samples harbor mutations in QRDR region of the *gyrA* gene**.**

1.10 Specific Aims

Our specific aims in this study are the following:

1. To properly identify *Salmonella* isolates
2. To serotype *Salmonella* isolates using the somatic "O" and flagellar "H" antigens.
3. To perform antimicrobial susceptibility testing by disk diffusion method.
4. To determine the presence of gene *gyrA* responsible for quinolone resistance using PCR.
5. To determine the specific mutation in the gene *gyrA* by sequencing.

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Materials and Methods

2.1 Specimens

A total of 151 non-typhoid *Salmonella* spp. were obtained from various clinical as well as food samples. Sixty-nine isolates were obtained from stool specimens, 2 isolates were obtained from blood specimens from patients with gastroenteritis. These samples were collected from outpatient clinics in Bethlehem and East Jerusalem hospitals between September 2006 and October 2007.

A total of 80 non-typhoid *Salmonella* spp. were obtained from contaminated food samples. These samples were provided by the Central Laboratory for Public Health at Ramallah between September 2005 and January 2007.

Stool samples were collected in a dry, clean, leak proof plastic container early in the course of infection. Each sample was labeled with the child's name, gender, age, and area of residence and sent to the laboratory within 30 minutes of sample collection.

2.2 Study population

This is a cross-sectional study conducted on specimens taken from children less than ten years of age with diarrhea. All specimens containing *Salmonella* spp. were included in this study. Most of the stool and blood specimens were collected from children ages from few days to less than 10 years. The specific age distribution of the patients is shown in Table 1. The source of the food samples is shown in Table 2 and figure 5.The geographical distributions of these patients are shown in Table 3.

Age (years)	Number of samples	Percentage
< 2	61	86.0
2 - 4	5	7.0
4 – 6	2	2.8
6 – 8	1	1.4
8 – 10	1	1.4
> 10	1	1.4

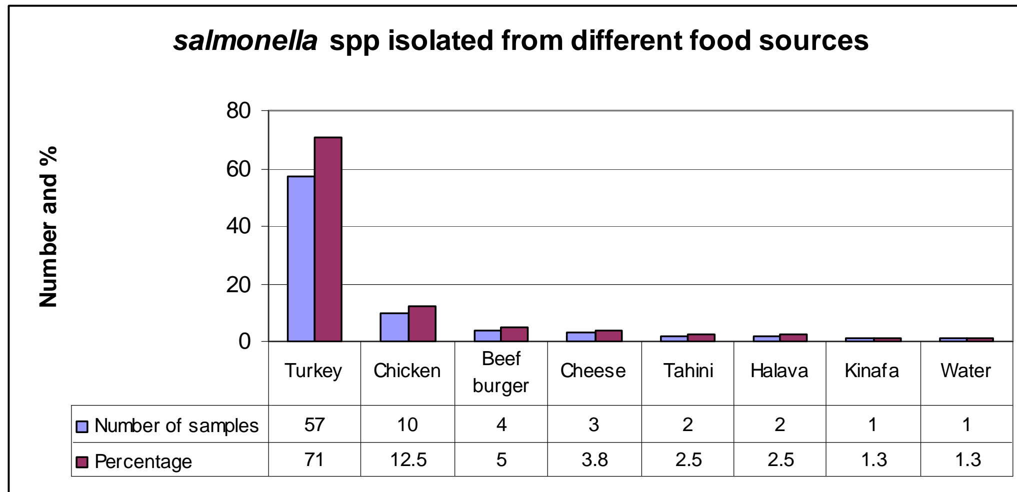
Table 1: Age distribution of patients with salmonellosis

Food Source)	Number of samples	Percentage
Turkey	57	71.0
Chicken	10	12.5
Beef burger	4	5.0
Cheese	3	3.8
Tahini	2	2.5
Halava	2	2.5
Kinafa	1	1.3
Water	1	1.3

Table 2: Predominance of *Salmonella* species in food samples

Area	Specimens	
City	**Stool**	**Blood**
Bethlehem	14	1
Jerusalem	0	1
Hebron	3	0
Beit sahour	6	0
Beit jala	6	0
Camp		
Dehesha	3	0
Aida	1	0
Azzeh	1	0
Aroub	2	0
Village		
Dar Salah	1	0
Nahhalin	1	0
Kisan	1	0
Urtas	1	0
Battir	1	0
Kharas	1	0
Wad Rahhal	2	0
Beidiyeh	3	0
Doura	2	0
Asakre	1	0
Al Rashaideh	1	0
Nouba	2	0
Beit Ummar	3	0
Sawahreh sharkeyh	1	0
Abu Injeim	1	0
Beit Fajjar	1	0
Ta'amreh	1	0
El Walajeh	2	0
Al khader	3	0
Aqbet Jaber	1	0
Tqou'	2	0
Beit Ula	1	0

Table 3: Geographic distribution of stool and blood specimens



	Turkey	Chicken	Beef burger	Cheese	Tahini	Halava	Kinafa	Water
Number of samples	57	10	4	3	2	2	1	1
Percentage	71	12.5	5	3.8	2.5	2.5	1.3	1.3

Figure 5: Predominance of *Salmonella* species in different food sources.

2.3 Laboratory diagnosis

2.3.1 Routine analysis

The stool sample is mixed well and examined for leukocytes, red blood cells and parasites.

2.3.2 Bacterial culture

Routinely, stool samples were inoculated on MacConkey agar (Oxoide LTD England), XLD agar (Oxoid LTD England), 5% blood agar (Oxoid LTD England), Campy agar (Oxoid LTD England), and in selenite broth (Biolife Italy).

Patients samples were incubated at 35°C for 24 hours, API20E kit was purchased from Biomerieux (France), and biochemical test were performed on suspected colonies *Salmonella* non-lactose fermenter on MacConkey and red with black center on XLD. Biochemical tests included urea broth (oxoide LTD England), Triple sugar iron (TSI) slants (Oxoid LTD England), SIM (Biolife Italy), Citrate agar slants (Biolife Italy), and mannitol (Merck Germany).

Confirmation and typing of *Salmonella* isolates was done with *Salmonella* O groups antisera (A, B, C, D, and E) purchased from Israel Ministry of Health using slide agglutination method according to the manufacturer guidelines.

2.3.3 *Salmonella* identification

Suspected colonies on XLD agar plates with the following biochemical reaction: urea: negative, TSI: alkaline/ acid with H2S, motile and gas producer. Conformation of the *Salmonella* isolates was done by typing with anti-sera specific for *salmonella* “O” and *Salmonella* “H” antigens.

Isolates identified as *salmonella* were subcultured on MacConkey agar plates; few colonies were inoculated into sterile eppendorf tubes in 1 ml Mueller Hinton broth containing 20% glycerol and stored at -4° C for further study. Antimicrobial susceptibility of *Salmonella* isolates was determined using disc diffusion method according to M-100-S14 Clinical Laboratory Standard Institute (CLSI) guidelines.

2.4 Antimicrobial susceptibility testing

Salmonella inoculated in sterile saline and adjusted to the turbidity of 0.5 McFarland Standard. Aliquots were spread on plates of Muller Hinton agar by sterile cotton swabs. Using aseptic techniques, sterile forceps was used to place a disc of antibiotics on the surface of MH-agar plate containing the inoculated bacterial isolate. Antibiotics which were used are shown in Table 4.

The plates were incubated at 37° C for 18 to 24 hours. The zone of inhibition around each antibiotic disc was measured with a Vernier Caliber to the nearest millimeter. Resistant (R), intermediate (I) or susceptible (S) zone diameters were recorded according to the recommendations and guidelines by CLSI.

Antibiotic Name	Concentration	Abbreviation
Ampicillin	10ug	AMP
Co-trimethoprim	25ug	SXT
Nalidixic acid	30ug	NA
Tetracycline	30ug	TE
Chloramphenicol	30ug	C
Erythromycin	15ug	E
Ciprofloxacin	5ug	CIP
Ceftriaxone	30ug	CRO
Oflaxcin	5ug	OFX
Gentamicin	10ug	CN

Table 4: Antibiotic discs used in the disc diffusion method for antimicrobial susceptibility testing of salmonella isolates

2.5 PCR detection of quinolones genes in *Salmonella*

2.5.1 Sample preparation:

Salmonella were subcultured on MacConkey agar plates and incubated at 37° C for 18 to 24 hours. A half-loopful of bacterial growth was removed from each plate and suspended in 200µl of sterile distilled water. The suspension was heated for 15 minutes at 95° C to release DNA template. The suspension was then centrifuged at 14,000 rpm for 2 min to precipitate undesired substances. Five µl of supernatant (cell lysate) containing the DNA template was added to 22 µl of PCR reaction mixture (Kariuki, *et al.,* 2004).

2.5.2 PCR mixture preparation:

Reaction conditions consisted of 50 ng of plasmid DNA and 100 nM concentration from the primer GYRA 1 and GYRA 2 were used (Table 5) in a buffer composed of 10 mM Tris-HCL (PH 8.3), 50 Mm KCL, 1.5 Mm $MgCl_2$, a 200 µM deoxynucleoide triphosphate mixture, and 1U of Taq polymerase in a final volume 100 µl.

Primer Name	Oligonucleotide sequence (5 -3)	Primer size (b)	product size (bp)
GYRA1	ATGAGCGACCTTGCGAGAGAAATTACACCG	30	630
GYRA2	TTCCATCAGCCCTTCAATGCTGATGTCTTC	30	

b: base

bp: base pair

Table 5: The *gyrA* primer used for PCR amplification and Sequencing of genes encoding for quinolone resistance (Kariuki, *et al.,* 2004).

2.5.3 DNA amplification

The amplification condition was set in the cycler to run 35 cycles, with denaturation at 94° C for 30 s, annealing at 55° C for 30s, and extension at 72° C for 30s, with a final extension step of 72° C for 10 min (Kenya, *et al*., 2004). 2% agarose gel electrophoresis was used to confirm that the PCR product obtained was correct. The PCR product was then digested by *Hinf*I (Fermentas).

2.6 **DNA** Restriction and Agarose Gel Electophoresis

2.6.1 ***Hinf*I restriction fragment length polymorphisms**

Restriction was achieved by adding 2 µl *Hinf*I (5 U), 2µl buffer to 16 µl PCR product in a total volume of 20µl and incubated up to 2 hours at 37 ^{o} C. The digested DNA was run on 2 % agarose gel. The size of fragments was then determined against a 50 bp ladder which was included in the run and the gel were photographed.

2.6.2 Agarose gel electrophoresis

The agarose gel was prepared as follows:

Agarose (2% w/v) were prepared in 25ml of Tris-EDTA buffer (TAE). The agarose was dissolved by heating then cooled to 50° C, and 2 µl of ethidium bromide (10mg/ml) was added. The gel was then poured into the electrophoresis cell. After solidification of the gel at room temperature, 1X TAE buffer was added to the electrophoresis cell, and the comb was carefully removed. 10 µl of PCR products was mixed with 2 µl of blue orange dye and then loaded. A 50 bp DNA marker was also included. The gel was run at 80V for 10 min then 120V for 30 minutes, viewed on a UV transilluminator and photographed.

2.7 DNA Sequencing:

The amplified PCR products from *Salmonella* isolates containing *gyrA* gene were purified using purification kit (Promega) and sequenced directly using ABI PRISM (310 genetic analyzer – Applied Biosystem) using big dye terminator VI.I cycle.

Results

Non-typhoid *Salmonella spp*. isolated from patients with gastroenteritis was obtained from several hospitals and outpatient clinics in Bethlehem and Jerusalem between September 2006 and October 2007. It is been noticed that most of the isolates were collected between June and October 2007 (Figure 6).

The isolates were kept at -20° C in Tryptic Soy Broth containing 20% glycerol. Non-typhoid *Salmonella* spp. was obtained from contaminated food samples were collected between September 2005 and January 2007. The bacterial isolates were then streaked on MacConkey and XLD agar plates to recover the *Salmonella* isolates

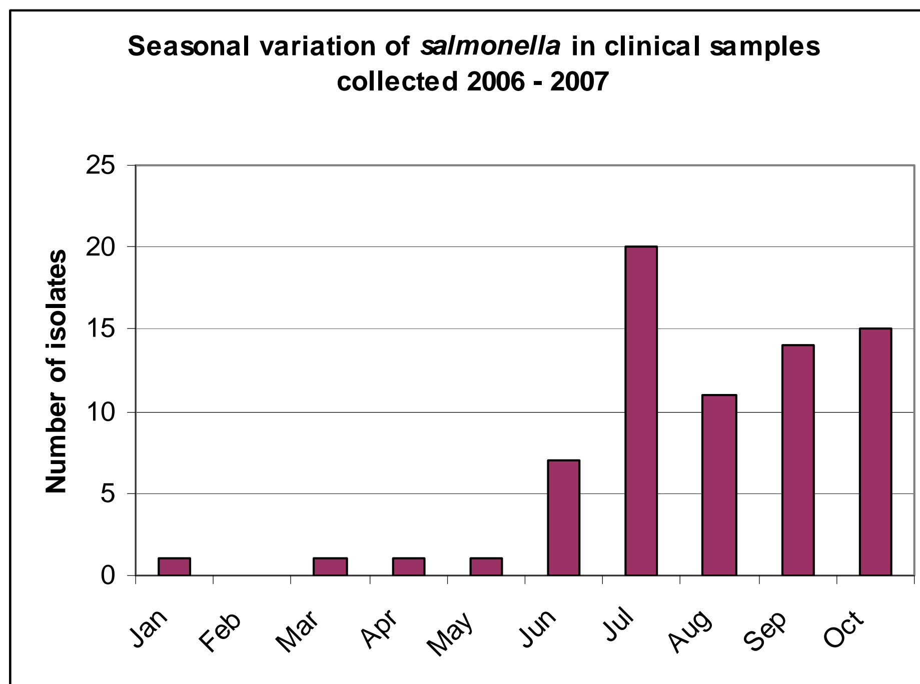


Figure 6: Seasonal variation of non-typhoid *Salmonella* isolates between Sep. 2006 and Oct. 2007

3.1 Biochemical Identification and Serotyping

The primary identification of *Salmonella* was done on the basis of non-lactose fermentation and hydrogen sulfide production on TSI slants and then by API20e. All the isolates obtained for this study were non-typhoid *Salmonella* spp. The most commonly encountered serotypes in both the clinical isolates (47/71) and food isolates (52/80) were *Salmonella* type O group C. The results of serotyping are summarized in table 6 and Figure 7.

	Clinical isolates		**Food Isolates**	
Serogroup	**Number of isolates**	**Percentage**	**Number of isolates**	**Percentage**
C	47	66.2	52	65.0
D	17	23.9	4	5.0
B	6	8.5	21	26.0
A	1	1.4	0	0.0
E	0	0.0	3	4.0
Total	71	100	80	100

Table 6: Predominant serogroups of non-typhoid *Salmonella* spp. obtained from clinical isolates and food sources.

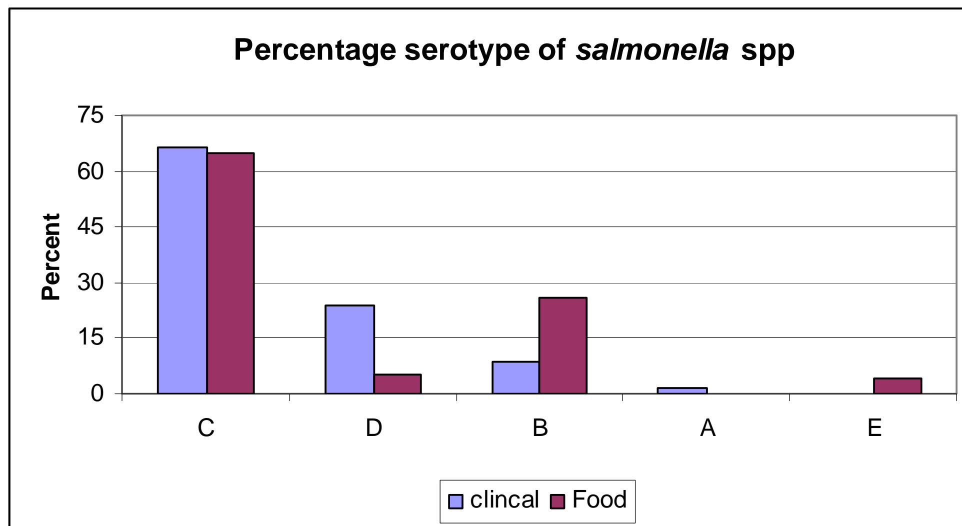


Figure 7: Predominance of *Salmonella* spp. serotypes isolated from food and clinical specimen.

3.2 Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed on a total of 10 antibiotics for both clinical and food isolates. The results for the antimicrobial susceptibility testing were interpreted according to the sensitive, intermediate, resistance system. The number of clinical and food isolates susceptible to both nalidixic acid and ciprofloxacin were found to be 27/71 and 41/80 respectively. The number of clinical and food isolates resistant to nalidixic acid but susceptible to ciprofloxacin were found to be 21/71 and 24/80 respectively. The number of clinical and food isolates resistant to both nalidixic acid and ciprofloxacin were found to be 21/71 and 12/80 respectively. The results of the antimicrobial susceptibility testing are summarized in Tables 7, 8, 9, 10, 11, 12, 13, and 14 and with their figures 8, 9, and 10.

Isolate ID	Identification	AMP	E	NA	cip	TE	GN
A5	S.O.C	R	R	R	R	R	R
B9	S.O.C	R	R	R	R	R	R
C1	S.O.C	R	R	R	R	R	R
C2	S.O.C	R	R	R	R	R	R
C3	S.O.C	S	R	R	R	R	S
C5	S.O.C	R	R	R	R	R	R
C6	S.O.C	R	R	R	R	R	R
D6	S.O.C	R	R	R	R	R	R
D7	S.O.C	R	R	R	R	R	R
D9	S.O.C	R	R	R	R	R	R
E3	S.O.C	R	R	R	R	R	R
E4	S.O.C	R	R	R	R	R	R
F2	S.O.C	R	R	R	R	R	R
G1	S.O.C	R	R	R	R	R	R
G6	S.O.C	R	R	R	R	R	R
H3	S.O.C	R	R	R	R	R	R
H6	S.O.C	R	R	R	R	R	R
H7	S.O.C	R	R	R	R	R	R
H8	S.O.C	R	R	R	R	R	R
H9	S.O.C	R	R	R	R	R	R
I4	S.O.C	R	R	R	R	R	R

Table 7: Results of antimicrobial susceptibility testing for clinical isolates of *Salmonella* with the serotype resistant to Na and Cip, S.O.C: *Salmonell*a O group C. S: sensitive, R: resistance, I: intermediate

Isolate ID	Identification	AMP	E	NA	Cip	TE	GN
A2	S.O.D	R	R	R	S	R	S
B2	S.O.D	S	R	R	S	S	S
B5	S.O.C	S	R	R	S	S	S
B6	S.O.C	R	R	R	S	R	S
B8	S.O.C	R	R	R	S	R	S
C4	S.O.C	R	R	R	S	R	S
D8	S.O.D	R	R	R	S	R	S
E1	S.O.D	R	R	R	S	S	S
F3	S.O.C	S	R	R	S	R	S
F7	S.O.C	S	R	R	S	R	S
F9	S.O.C	S	R	R	S	R	S
G3	S.O.C	S	R	R	S	S	S
G7	S.O.C	S	R	R	S	S	S
G8	S.O.C	S	R	R	S	R	S
G9	S.O.C	S	R	R	S	R	S
H1	S.O.C	S	R	R	S	R	S
H2	S.O.C	S	R	R	S	S	S
H4	S.O.C	S	R	R	S	R	S
H5	S.O.C	S	R	R	S	S	S
I3	S.O.C	S	R	R	S	S	S
I5	S.O.B	R	R	R	S	R	S

Table 8: Results the antimicrobial susceptibility testing for clinical isolates of *Salmonella* with the serotype resistant to NA but sensitive to Cip S.O.C : *Salmonella* O group C, S.O.B: *Salmonella* O group B, S.O.D: *Salmonella* O group D. S: sensitive, R: resistance, I: intermediate.

Isolate ID	Identification	AMP	E	NA	Cip	TE	GN	CRO
A1	S.O.D	R	R	S	S	S	S	S
A3	S.O.D	R	R	S	S	S	S	S
A4	S.O.D	R	R	S	S	S	S	S
A9	S.O.D	R	R	S	S	S	S	S
B1	S.O.D	R	R	S	S	S	S	S
B3	S.O.C	R	R	S	S	I	S	S
B4	S.O.D	R	R	S	S	S	S	S
C7	S.O.B	R	R	S	S	R	S	S
C8	S.O.B	R	R	S	S	R	S	S
D1	S.O.C	R	R	S	S	S	S	S
D2	S.O.C	R	R	S	S	S	S	S
D3	S.O.C	R	R	S	S	R	S	S
D4	S.O.B	S	R	S	S	S	S	S
D5	S.O.D	R	R	S	S	R	S	S
E5	S.O.C	S	R	S	S	S	S	S
E6	S.O.C	S	R	S	S	S	S	S
E7	S.O.D	I	R	I	S	R	S	S
E8	S.O.A	S	R	S	S	I	S	S
E9	S.O.D	I	R	I	S	R	S	S
F1	S.O.D	S	R	S	S	I	S	S
F4	S.O.D	S	R	S	S	S	S	S
F5	S.O.D	S	R	S	S	S	S	S
F6	S.O.D	S	R	S	S	S	S	S
F8	S.O.C	S	R	S	S	S	S	S
G2	S.O.C	I	R	S	S	S	S	S
G4	S.O.C	S	R	S	S	S	S	S
G5	S.O.B	R	R	S	S	R	R	S
I1	S.O.D	S	R	S	S	S	S	S
I2	S.O.C	R	R	S	S	R	S	S

Table 9: Results of antimicrobial susceptibility testing for clinical isolates of *Salmonella* with the serotype sensitive to both Na and Cip, S.O.C: *Salmonella* O group C, S.O.B: *Salmonella* O group B, S.O.D: *Salmonella* O group D, S.O.A: *Salmonella* O group A. S: sensitive, R: resistance, I: intermediate.

Isolate ID	SPECIMEN	Identification	Amp	E	TE	NA	Cip	GE
FA4	TURKY	S.O.C	R	R	R	R	R	R
FA5	TURKY	S.O.C	R	R	R	R	R	R
FA7	CHICKEN	S.O.C	R	R	R	R	R	R
FB1	TURKY	S.O.C	R	R	R	R	R	R
FB2	TURKY	S.O.C	R	R	R	R	R	R
FE4	TURKY	S.O.C	R	R	R	R	R	I
FF5	TURKY	S.O.C	R	R	R	R	R	I
FF6	TURKY	S.O.C	R	R	R	R	R	I
FF9	TURKY	S.O.C	R	R	R	R	R	I
FG2	TURKY	S.O.C	R	R	R	R	R	R
FG8	CHICKEN	S.O.C	R	R	R	R	R	R
FI7	CHICKEN	S.O.C	R	R	R	R	R	R

Table 10: Results of antimicrobial susceptibility testing of *Salmonella* isolates from food samples with the serotype resistant to both Na and Cip, S.O.C: *Salmonella* O group C. S: sensitive, R: resistance, I: intermediate.

Isolate ID	SPECIMEN	Identification	Amp	E	TE	NA	Cip	GE
FF4	TURKY	S.O.B	R	R	R	R	S	S
FG1	TURKY	S.O.B	R	R	R	R	S	S
FG9	TURKY	S.O.B	R	R	R	R	S	S
FH3	CHICKEN	S.O.C	S	R	R	R	S	S
FH4	TURKY	S.O.B	S	R	S	R	S	S
FH5	TURKY	S.O.B	R	R	R	R	S	S
FH8	TURKY	S.O.D	R	R	R	R	S	S
FH9	TURKY	S.O.C	S	R	S	R	S	S
FI1	TURKY	S.O.C	R	R	R	R	S	S
FE7	TURKY	S.O.B	R	R	R	R	S	S
FE5	CHICKEN	S.O.B	S	R	R	R	S	S
FD3	TURKY	S.O.B	R	R	R	R	S	S
FD1	TURKY	S.O.B	R	R	R	R	S	S
FC2	TURKY	S.O.B	R	R	R	R	S	S
FC3	TURKY	S.O.B	R	R	R	R	S	S
FC4	TURKY	S.O.B	R	R	R	R	S	S
FB9	TURKY	S.O.B	R	R	R	R	S	S
FA8	CHICKEN	S.O.C	R	R	S	R	S	S
FA9	TURKY	S.O.B	R	R	R	R	S	S
FB3	TURKY	S.O.C	R	R	R	R	S	S
FB4	TURKY	S.O.C	R	R	R	R	S	S
FB7	TURKY	S.O.C	S	R	R	R	S	S
FA1	TURKY	S.O.B	R	R	R	R	S	S
FA2	CHICKEN	S.O.C	S	R	R	R	S	S

Table 11: Results of antimicrobial susceptibility testing of *Salmonella* isolates from food samples with the serotype resistant to Na but sensitive to Cip, S.O.C: *Salmonella* O group C, S.O.B: *Salmonella* O group B, S.O.D: *Salmonella* O group D. S: sensitive, R: resistance, I: intermediate

Isolate ID	SPECIMEN	Identification	Amp	E	TE	NA	cip	GE
FI2	TURKY	S.O.C	R	R	R	S	S	S
FI3	TURKY	S.O.C	S	R	R	S	S	S
FI4	KONAFA	S.O.C	S	R	S	S	S	S
FI5	TIHENIA	S.O.E	S	R	S	S	S	S
FI6	TIHENIA	S.O.E	S	R	S	S	S	S
FI8	TURKY	S.O.D	S	R	R	S	S	S
FH7	HALAWA	S.O.C	S	R	S	S	S	S
FH6	HALAWA	S.O.C	S	R	R	S	S	S
FH1	TURKY	S.O.B	S	R	S	S	S	S
FH2	CHICKEN	S.O.E	S	R	R	S	S	S
FG3	TURKY	S.O.B	S	R	I	S	S	S
FG4	TURKY	S.O.B	S	R	R	S	S	S
FG5	BEEF MEAT	S.O.C	S	R	R	S	S	S
FG6	BEEF MEAT	S.O.C	S	R	I	S	S	S
FG7	CHICKEN	S.O.D	S	R	R	S	S	S
FF7	TURKY	S.O.C	S	R	R	S	S	S
FF8	TURKY	S.O.C	R	R	R	S	S	S
FE8	TURKY	S.O.C	S	R	R	S	S	S
FE9	TURKY	S.O.B	S	R	R	S	S	S
FF1	TURKY	S.O.C	S	R	R	S	S	S
FF2	TURKY	S.O.C	S	R	S	S	S	S
FF3	WATER	S.O.C	S	R	R	S	S	S
FE6	CHICKEN	S.O.C	S	R	R	S	S	S
FD5	TURKY	S.O.C	R	R	R	S	S	S
FD6	TURKY	S.O.C	S	R	R	S	S	S
FD7	TURKY	S.O.C	S	R	R	S	S	S
FD8	TURKY	S.O.C	S	9	R	S	S	S
FD9	TURKY	S.O.C	S	R	R	S	S	S
FE1	TURKY	S.O.C	R	R	R	I	S	S
FE2	CHEESE	S.O.C	R	R	R	S	S	S
FE3	CHEESE	S.O.C	S	R	R	S	S	S
FC5	BEEF burger	S.O.C	S	R	S	S	S	S
FC6	BEEF burger	S.O.C	S	R	S	S	S	S
FC7	CHEESE	S.O.D	S	R	S	S	S	S
FC8	TURKY	S.O.C	R	R	S	I	S	S
FC9	TURKY	S.O.C	R	R	R	S	S	S
FD2	TURKY	S.O.C	R	R	R	S	S	S
FD4	TURKY	S.O.C	R	R	R	I	S	S
FB8	TURKY	S.O.B	S	R	R	S	S	S
FC1	TURKY	S.O.C	S	R	R	S	S	S
FB5	TURKY	S.O.C	R	R	R	S	S	S
FB6	TURKY	S.O.B	S	R	R	S	S	S
FA3	TURKY	S.O.C	R	R	R	S	S	S
FA6	TURKY	S.O.C	S	R	R	S	S	S

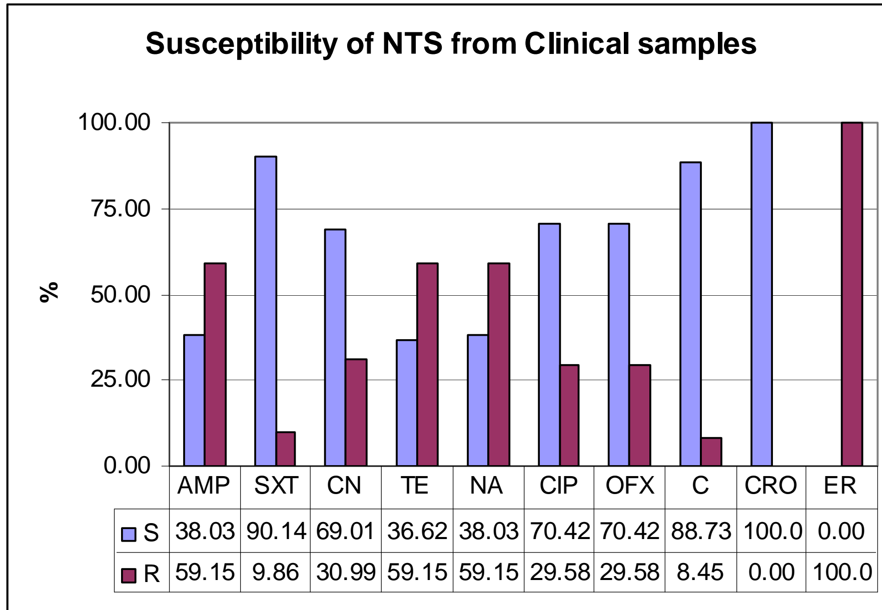
Table 12: Results of antimicrobial susceptibility testing of *Salmonella i*solates fom food samples with the serotype sensitive to both Na and Cip, S.O.C: *Salmonella* O group C, S.O.B: *Salmonella* O group B, S.O.D: *Salmonella* O group D, S.O.E: *Salmonella* O group E. S: sensitive, R: resistance, I: intermediate

	Clinical Isolates			Food		
Drugs	**S**	**I**	**R**	**S**	**I**	**R**
AMP	27	2	42	39	0	41
CRO	71	0	0	80	0	0
C	63	2	6	76	0	4
SXT	64	0	7	76	0	4
CN	49	0	22	68	4	8
TE	26	3	42	13	3	64
ER	0	0	71	0	0	80
NA	27	2	42	41	3	36
CIP	50	0	21	65	3	12
OFX	50	0	21	65	3	12

Table 13: Summary of the antimicrobial susceptibility testing of the *Salmonella spp*. performed on the clinical and food isolates. S: sensitive, R: resistance, I: intermediate

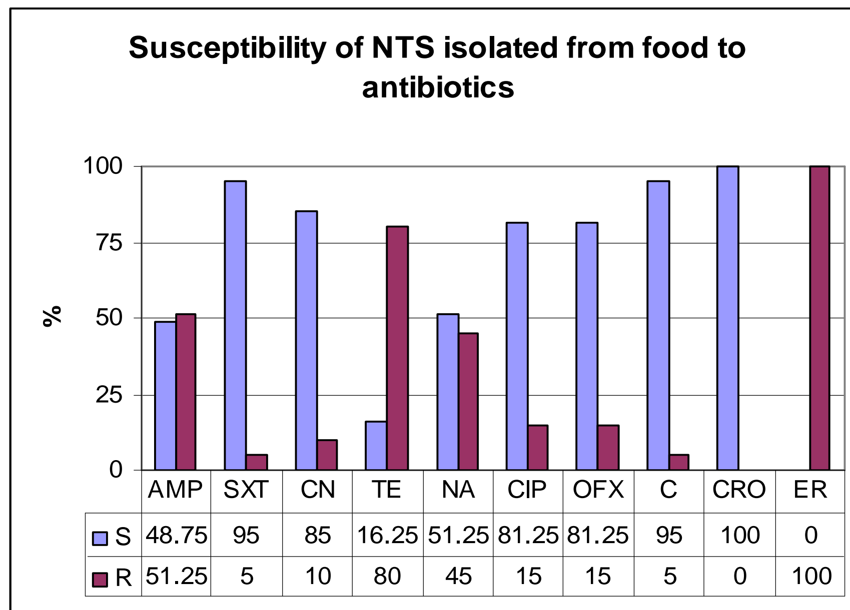
Quinolones	Pattern	Clinical	Food
NA / CIP	SS	27	41
NA / CIP	RS	21	24
NA / CIP	RR	21	12

Table 14: Summary of antimicrobial susceptibility for the clinical and food isolates in terms of susceptibly and resistance to both nalidixic acid and ciprofloxacin.



	AMP	SXT	CN	TE	NA	CIP	OFX	C	CRO	ER
S	38.03	90.14	69.01	36.62	38.03	70.42	70.42	88.73	100.0	0.00
R	59.15	9.86	30.99	59.15	59.15	29.58	29.58	8.45	0.00	100.0

Figure 8: Antimicrobial susceptibility of NTS obtained from clinical specimens.



	AMP	SXT	CN	TE	NA	CIP	OFX	C	CRO	ER
S	48.75	95	85	16.25	51.25	81.25	81.25	95	100	0
R	51.25	5	10	80	45	15	15	5	0	100

Figure 9: Antimicrobial susceptibility of NTS obtained from foods.

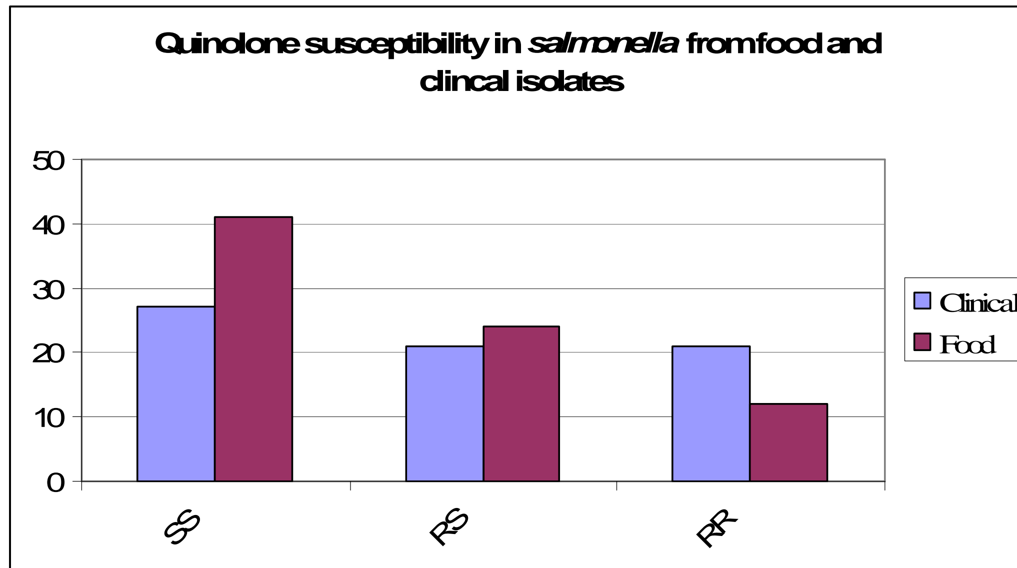


Figure 10: Patterns of susceptibility of NTS isolated from food and clinical specimens to quinolones.

3.3 PCR Results:

The *gyrA* gene was amplified by PCR. The PCR products were analyzed on agarose gel electrophoreasis. A sharp band with 630 bp size measured against 50 bp ladder was obtained. Representative photographs of the gels are shown below in figures 11, (A, B, C and D). The PCR product was subjected to restriction by *Hinf* I enzyme. Various size bands were obtained depending on the pattern of susceptibility or resistance of *Salmonella* isolates to nalidixic acid and ciprofloxacin. Depending on susceptibility to these two antibiotics, 3 patterns were identified: sensitive to both (SS), resistant to nalidixic acid but susceptible to ciprofloxacin (RS) and resistant to both (RR).

Restricted and amplified DNA from the *Salmonella* isolates with the SS pattern revealed 3 major bands of 250 bp, 150 bp and 100 bp bands. A faintly appearing

fourth band of about 130 bp was also seen. Identical results were obtained with the RS group. With RR pattern, two major bands of 350 bp and 150 bp were evident. A faintly appearing third band of 130 bp was also seen in Table 15. The results of these experiments are shown in Figures 12 (E, F, G, and H)

Quinolones	**Pattern**	**Rescriction band sizes**		
NA / CIP	SS	250 bp	150 bp	100 bp
NA / CIP	RS	250 bp	150 bp	100 bp
NA / CIP	RR	350 bp	150 bp	

Table 15: Restricted and amplified DNA from the *Salmonella* with SS, RS, and RR patterns

3.4 Sequencing Results

Representative samples for the three patterns identified previously as SS, RS and RR were sequenced. The sequence obtained for the SS pattern was normal without any mutation as shown in figure 13. The sequence obtained with the RS pattern revealed a mutation at position 87. A substitution of G to A (GAC became TAC) that has resulted in the replacement of D (Aspartic acid) amino acid by Y (Tyrosine) as shown in figure 14 The sequence obtained with the RR pattern revealed two mutations at positions 83 and 87. At position 83, a substitution of C to T (TCC became TTC) that has resulted in the replacement of S (Serine) by F (Phenylalanine). At position 87, the same type of mutation occurred as in the RS pattern, a substitution of G to A (GAC became TAC) that has resulted in the replacement of D (Aspartic acid) amino acid by Y (Tyrosine). The results of the sequencing are shown in Figure 15.

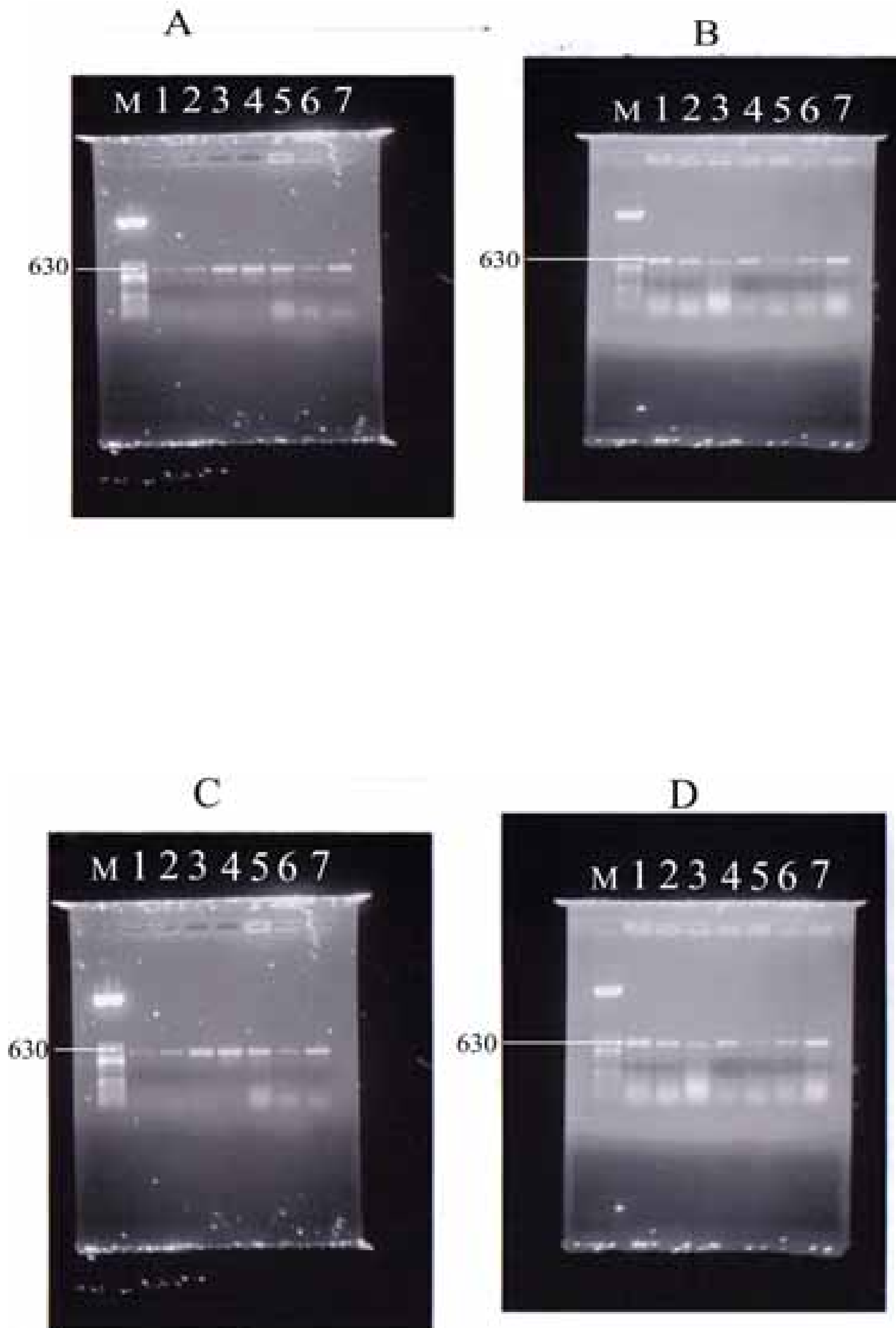


Figure 11 (A,B,C,and D) gel electrophoresis for the primary PCR products against 50 bp DNA ladder. A band size of 630 bp was obtained for the *gyrA* gene.

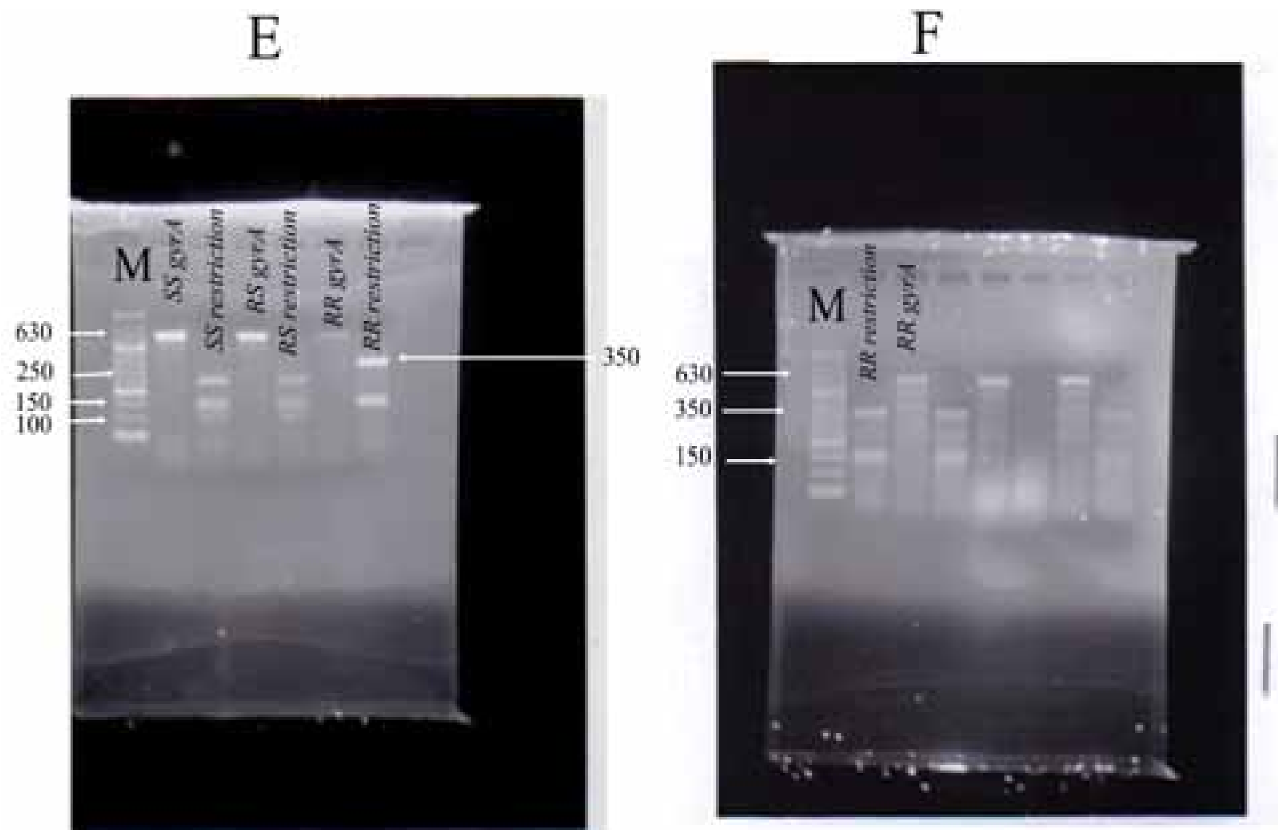


Figure 12: Represent the restriction fragments of *gyrA* gene produced by *Hinf*I.

E: Lane 1 (M), 50 bp ladder, lane 2, *gyrA* gene from SS pattern, lane 3 restriction pattern in lane 2, lane 4, *gyrA* for RS pattern, lane 5, restriction pattern in lane 4, lane 6 *gyrA* from RR pattern, lane 7, restriction pattern in lane 6.

F: lane 1 (M) 50 bp ladder, lane 2 restriction pattern in lane 3, Lane 3, *gyrA* gene from RR pattern, 4 and 8 are the restriction pattern, lanes 5 and 7 are the *gyrA* gene for RR pattern.

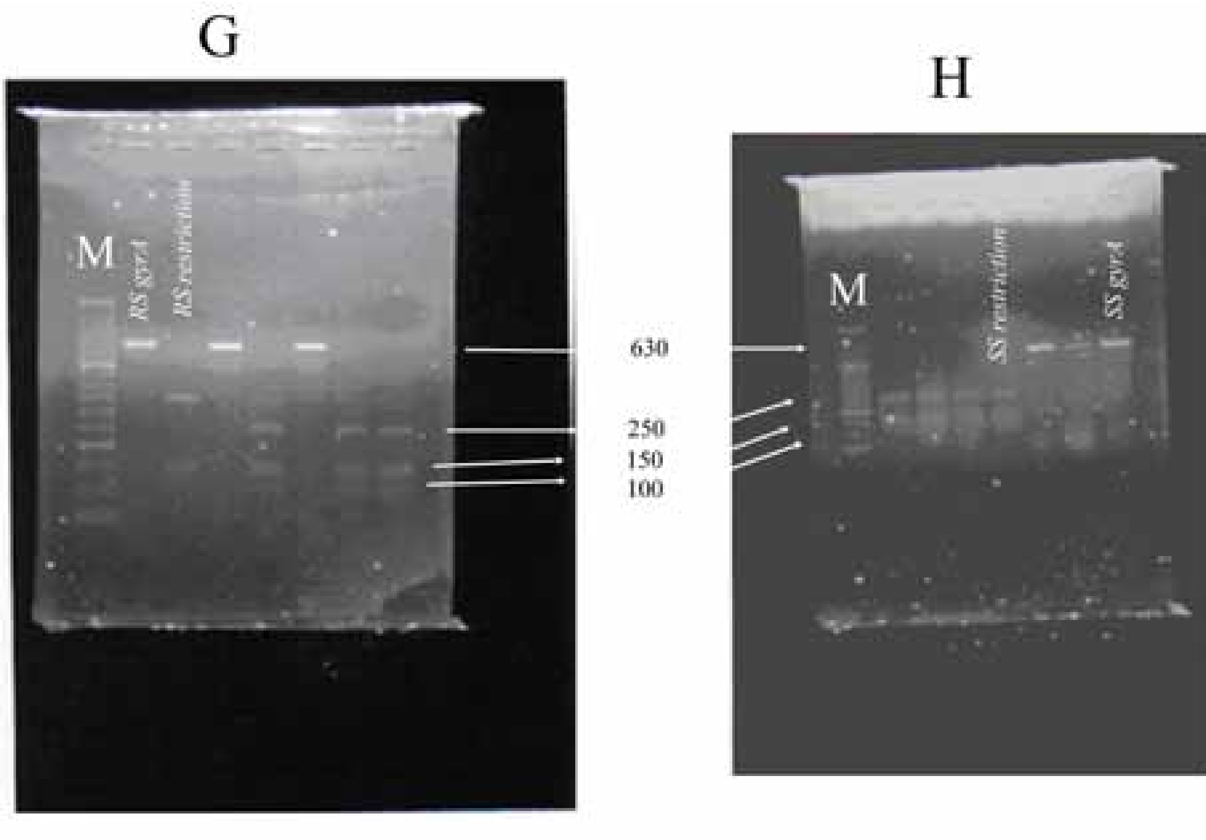


Figure12: Represent the restriction fragments of *gyrA* gene produced by *Hinf* I.

G: Lane 1(M), 50 bp ladder, lane 2, *gyrA* gene from RS pattern, lane 3 restriction pattern in lane 2, lane 4 and 6 are *gyrA* from RS pattern.

H: *gyrA* for the SS pattern, lane 1 (M), 50 bp ladder, Lanes 6, 7 and 8 for *gyrA* gene from SS pattern. Lanes 2, 3, 4 and 5 are the restriction pattern.

```
Score =  531 bits (276),  Expect = 7e-148
Identities = 276/276 (100%), Gaps = 0/276 (0%)
Strand=Plus/Minus

CDS: Putative 1        1       S  Y  L  D  T  A  M  S  V  I  V  G  R  A  L  P  D  V  R  D
Query                  25     CTCCTATCTGGATTATGCGATGTCGGTCATTGTTGGCCGTGCGCTGCCGGATGTCCGAGA  84
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21520  CTCCTATCTGGATTATGCGATGTCGGTCATTGTTGGCCGTGCGCTGCCGGATGTCCGAGA  21461
CDS:DNA gyrase, subu   20      S  Y  L  D  T  A  M  S  V  I  V  G  R  A  L  P  D  V  R  D

CDS: Putative 1        21      G  L  K  P  V  H  R  R  V  L  T  A  M  N  V  L  G  N  D  W
Query                  85     TGGCCTGAAGCCGGTACACCGTCGCGTACTTTACGCCATGAACGTATTGGGCAATGACTG  144
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21460  TGGCCTGAAGCCGGTACACCGTCGCGTACTTTACGCCATGAACGTATTGGGCAATGACTG  21401
CDS:DNA gyrase, subu   40      G  L  K  P  V  H  R  R  V  L  Y  A  M  N  V  L  G  N  D  W

CDS: Putative 1        41      N  K  A  Y  K  K  S  A  R  V  V  G  D  V  I  G  K  Y  H  P
Query                  145    GAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCATCC  204
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21400  GAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCATCC  21341
CDS:DNA gyrase, subu   60      N  K  A  Y  K  K  S  A  R  V  V  G  D  V  I  G  K  Y  H  P

CDS: Putative 1        61      H  G  D  S  A  V  Y  D  T  I  V  R  M  A  Q  P  F  S  L  R
Query                  205    CCACGGCGATTCCGCAGTGTATGACACCATCGTTCGTATGGCGCAGCCATTCTCGCTGCG  264
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21340  CCACGGCGATTCCGCAGTGTATGACACCATCGTTCGTATGGCGCAGCCATTCTCGCTGCG  21281
CDS:DNA gyrase, subu   80      H  G  D  S  A  V  Y  D  T  I  V  R  M  A  Q  P  F  S  L  R

CDS: Putative 1        81      Y  M  L  V  D  G  Q  G  N  F  G  S
Query                  265    TTACATGCTGGTGGATGGTCAGGGTAACTTCGGTTC  300
                              ||||||||||||||||||||||||||||||||||||
Sbjct                  21280  TTACATGCTGGTGGATGGTCAGGGTAACTTCGGTTC  21245
CDS:DNA gyrase, subu   100     Y  M  L  V  D  G  Q  G  N  F  G  S
```

Figure 13: The sequence for SS pattern was normal without any mutation.

```
Strand=Plus/Minus

CDS: Putative 1        1      S  Y  L  D  Y  A  M  S  V  I  V  G  R  A  L  P  D
Query                  21     CTCCTATCTGGATTATGCGATGTCGGTCATTGTTGGCCGTGCGCTGCCGGAT
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21520  CTCCTATCTGGATTATGCGATGTCGGTCATTGTTGGCCGTGCGCTGCCGGAT
CDS:DNA gyrase, subu   20     S  Y  L  D  Y  A  M  S  V  I  V  G  R  A  L  P  D

CDS: Putative 1        21     G  L  K  P  V  H  R  R  V  L  Y  A  M  N  V  L  G
Query                  81     TGGCCTGAAGCCGGTACACCGTCGCGTACTTTACGCCATGAACGTATTGGGC
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21460  TGGCCTGAAGCCGGTACACCGTCGCGTACTTTACGCCATGAACGTATTGGGC
CDS:DNA gyrase, subu   40     G  L  K  P  V  H  R  R  V  L  Y  A  M  N  V  L  G

CDS: Putative 1        41     N  K  A  Y  K  K  S  A  R  V  V  G  D  V  I  G  K
Query                  141    GAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAA
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21400  GAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAA
CDS:DNA gyrase, subu   60     N  K  A  Y  K  K  S  A  R  V  V  G  D  V  I  G  K

CDS: Putative 1        61     H  G  D  S  A  V  Y  Y  T  I  V  R  M
Query                  201    CCACGGCGATTCCGCAGTGTATTACACCATCGTTCGTATG  240
                              ||||||||||||||||||||||| ||||||||||||||||
Sbjct                  21340  CCACGGCGATTCCGCAGTGTATGACACCATCGTTCGTATG  21301
CDS:DNA gyrase, subu   80     H  G  D  S  A  V  Y  D  T  I  V  R  M
```

Figure14. The sequence for RS pattern with a mutation at position 87, (GAC became TAC.)

```
Score = 487 bits (253), Expect = 1e-134
Identities = 257/259 (99%), Gaps = 0/259 (0%)
Strand=Plus/Minus

CDS: Putative 1        1       Y  A  M  S  V  I  V  G  R  A  L  P  D  V  R  D  G  L  K
Query                  42     ATTATGCGATGTCGGTCATTGTTGGCCGTGCGCTGCCGGATGTCCGAGATGGCCTGAAGC  101
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21509  ATTATGCGATGTCGGTCATTGTTGGCCGTGCGCTGCCGGATGTCCGAGATGGCCTGAAGC  21450
CDS:DNA gyrase, subu   23     D  Y  A  M  S  V  I  V  G  R  A  L  P  D  V  R  D  G  L  K

CDS: Putative 1        20     P  V  H  R  R  V  L  Y  A  M  N  V  L  G  N  D  W  N  K  A
Query                  102    CGGTACACCGTCGCGTACTTTACGCCATGAACGTATTGGGCAATGACTGGAACAAAGCCT  161
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21449  CGGTACACCGTCGCGTACTTTACGCCATGAACGTATTGGGCAATGACTGGAACAAAGCCT  21390
CDS:DNA gyrase, subu   43     P  V  H  R  R  V  L  Y  A  M  N  V  L  G  N  D  W  N  K  A

CDS: Putative 1        40     Y  K  K  S  A  R  V  V  G  D  V  I  G  K  Y  H  P  H  G  D
Query                  162    ATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCATCCCCACGGCGATT  221
                              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21389  ATAAAAAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCATCCCCACGGCGATT  21330
CDS:DNA gyrase, subu   63     Y  K  K  S  A  R  V  V  G  D  V  I  G  K  Y  H  P  H  G  D

CDS: Putative 1        60     F  A  V  Y  Y  T  I  V  R  M  A  Q  P  F  S  L  R  Y  M  L
Query                  222    TCGCAGTGTATTACACCATCGTTCGTATGGCGCAGCCATTCTCGCTGCGTTACATGCTGG  281
                               ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                  21329  CCGCAGTGTATGACACCATCGTTCGTATGGCGCAGCCATTCTCGCTGCGTTACATGCTGG  21270
CDS:DNA gyrase, subu   83     S  A  V  Y  D  T  I  V  R  M  A  Q  P  F  S  L  R  Y  M  L

CDS: Putative 1        80     V  D  G  Q  G  N  X
Query                  282    TGGATGGTCAGGGTAACTT  300
                              |||||||||||||||||||
Sbjct                  21269  TGGATGGTCAGGGTAACTT  21251
CDS:DNA gyrase, subu   103    V  D  G  Q  G  N  F
```

Figure15: The sequence for RR pattern with two mutations at position 83 and 87, (TCC became TTC and GAC became TAC)

Discussion

Nontyphoidal salmonellosis is a significant public health problem worldwide. Most of the cases are found in the developing countries. In the United States, *Salmonella* is considered to be the most common enteric pathogen and most frequently isolated from children younger than 5 years of age (CDC 2004, CDC 2005 and CDC 2006). In US, an estimated 1.4 million cases occur per year with 15,000 hospitalization and 400 fatal cases (Stevenson*,* 2007).

In this study, it was found that the most commonly contaminated foods with salmonellae were turkey (71%), chickens (13%), ground meat (5%) and cheese (4%). Serotyping of the non-typhoid *Salmonella spp*. isolated from food revealed that groups C (52/80) was the most common followed by group B (21/80). Non-typhoid *Salmonella spp*. was also isolated from clinical specimens. The majority of the isolates were from children less than 4 years of age (95%). Group C S*almonella* was the most common (47/71) followed by group D (17/71).

Resistance of *Salmonella* to antibiotics has increased in several parts of the world posing a problem to healthcare systems worldwide. Fluoroquinolones are the most commonly used antimicrobial agents for the treatment of severe *Salmonella* infection in adults (Stevenson*.,* 2007; Hopkins, *et al*., 2005). Several studies have suggested that the use of fluoroquinolones in veterinary medicine contributes to the emergence and dissemination of nalidixic acid resistance in *Salmonella* among food animals, which may be transmitted to human. These data contributed to the decision by FDA in the United States to discontinue the use of fluoroquinolones in chickens and turkeys as of September 2005 (Stevenson*.,* 2007). In Europe, the rate of quinolone resistance

is also very high (Threlfall, *et al.,* 2003). Antimicrobial susceptibility testing performed according to CLSI guidelines, revealed a high rate of resistance to many antibiotics. The highest resistance rate was associated with nalidixic acid. Resistance to nalidixic acid among isolates of NTS in Belgium had doubled during the period of 2000 - 2003 to 84% (Stevenson, *et al.,* 2007). Other studies resistance to nalidixic acid, among other prevalent *Salmonella* serovars in Israel, was less common of 54% (Mates, *et al.,* 2002).

In our study, resistance to nalidixic acid in clinical and food samples was found to be 59.2% and 45% respectively. This rate is comparable to the rate reported in Israel. In the United States, Resistance of NTS to nalidixic was monitored from 1996 to 2003. Resistance to nalidixic acid in the year 2003 was 2.3 % as compared to 0.4% in 1996, a 7-fold increase. A study conducted in Mexico over 3 years period, revealed the presence of *Salmonella* in high percentage in pork and poultry (Zaidi, *et al.,* 2006). These strains of *Salmonella* were resistant to nalidixic acid (27%) but not to ciprofloxacin. In Vietnam, a similar study evaluated drug resistance of *Salmonella* from pork and poultry, it reported a nalidixic acid resistance of 18.7% but none of these isolates was found to resistant to ciprofloxacin (Hao, *et al.,* 2007).

In this study a high level resistance to ciprofloxacin was found in both clinical specimens as well as in food samples, 30 % and 15% respectively. Several studies conducted on quinolone resistance in food did not report any resistance to ciprofloxacin (Hao, *et al.,* 2007; Weill, *et al.,* 2006).

The resistance of *Salmonella spp*. to ampicillin, tetracycline trimethoprim-sulphamethoxazole, ceftriaxone and gentamicin was evaluated (Stevenson, 2007). In our study, resistance to ampicillin by *Salmonella* isolated from food and clinical samples were found to be 51.3% and 59.2 % respectively. A study conducted in Korea (Sang Ho Choi, *et al.,* 2005) on human isolates of non-typhoid *Salmonella* revealed an ampicillin resistance of 39.1%, much lower than our results. A Brazilian study revealed a resistance to ampicillin of 7.0% (Oplustil, *et al.,* 2001) as compared to resistance rates of 60.3% in Argentina, 20.0% in Coloumbia, and 16.7% in Mexico (Oplustil, *et al.,* 2001). Resistance to tetracycline was found to be high in this study (59.2% for clinical isolates and 80% for food isolates). In a Korean study (Choi, *et al.,* 2005), they reported a resistance rate of 69%.

Resistance to other antibiotics tested in our study included trimethoprim sulfa-methaxozole (SXT), gentamicin and ceftriaxone. Resistance to these antibiotics in the human isolates was found to be 9.9%, 30.9%, and 0 % respectively. In a similar study conducted in Korea, the pattern of resistance to these antibiotics was 15.2%, 45.7% and 0% respectively. Resistance to ampicillin seems to be a big problem world wide for both non-typhoid as well as typhoidal *Salmonella* isolates. Outbreaks of multidrug resistance in *S. typhi* to ampicillin, SXT and chloramphenicol were reported in India, Arabian Gulf states, Phillipines and South Africa (Kariuki, *et al*., 2004).

Quinolone Resistance of *Salmonella enterica* from human and poultry is of major concern to health care facilities. Studies on the QRDR region revealed that mutations in *gyrA* can be sufficient to cause high-level resistance to nalidixic acid in *Salmonella* (Giraud, *et al.,* 2006).

In this study we screened clinical specimens as well as food samples for mutations in the *gyrA* gene. The reason is to detect the existence of high incidence of resistance among the isolates. Initially the *gyrA* gene was amplified and restricted and then resolved on agarose gel electrophoresis. Representative bands on all groups were then sequenced to definitely identify the presence and nature of mutations.

Amplification of the *gyrA* gene and subsequent agarose gel electrophoresis revealed a band of 630 bps. Restriction of the PCR products by *Hinf* I resulted in various bands that correlate with the pattern of susceptibility and resistance to nalidixic acid and ciprofloxacin. Three different patterns were identified in terms of nalidixic acid /ciprofloxacin: SS, RS and RR.

Similar to the results of this study, for the different serovars with the Asp 87 Tyr substitution, the nalidixic acid MICs were between 64 and 512 µg/ml and ciprofloxacin MICs were between 0.125 and 1 µg /ml, lower MICs than for strains with other mutations in the codons of Asp87 or Ser83 (Galan and Wolf-Watz, 2006). Results obtained in our study for the amplification of the *gyrA* gene gave a band size of 630 bp for all *Salmonella* isolates. Review of literature revealed that our study are comparable to other studies done in India (Brown, *et al.,* 1996), the United Kingdom (Brown and Shanahan 1996),) and Kenya (Kariuki, *et al.*, 2004). The primers we used in this study were identical to those used in these papers.

Restriction by *Hinf* I enzyme resulted in different band sizes depending on the pattern of susceptibility to nalidixic acid and ciprofloxacin. The SS and RS patterns of the NTS isolates revealed 3 major bands of 250 bp, 150 bp and 100 bp. A faintly

appearing fourth band of about 130 bp was also seen. With the RR pattern, two major bands of 350 bp and 150 bp were evident. A faintly appearing third band of 130 bp was also seen. Review of literature revealed similar results to those obtained by us. A similar study conducted in India (Brown and Thomson, 1996). On *S. typhi*, susceptible to both nalidixic acid and ciprofloxacin, resulted in 4 bands of 246 bp, 99 bp, 149 bp and 126 bp. The number of bands and their sizes obtained for the *S. typhi* strains in the Indian study is almost identical to the number of bands and their sizes obtained in our study. Another study was conducted in Santiago Chile (Martin, *et al.,* 2004) *Salmonella* isolated from poultry. Although the primers used were different, the outcomes of their study were similar to those obtained by us. The outcomes of these experiments indicated a mutation on the *gyrA* gene at positions 83 or 87 depending on the pattern of resistance.

Representative samples for the three patterns identified previously as SS, RS and RR were sequenced. The sequence obtained for the SS pattern was normal without any mutation. The sequence obtained with the RS pattern revealed a mutation at position 87. A substitution of G to T (GAC became TAC) that has resulted in the replacement of aspartic acid to tyrosine. The sequence obtained with the RR pattern revealed two mutations at positions 83 and 87. At position 83, a substitution of C to T (TCC became TTC) that has resulted in the replacement of Serine by phenylalanine. At position 87, the same type of mutation occurred as in the RS pattern, a substitution of G to A (GAC became TAC) that has resulted in the replacement of aspartic acid by tyrosine. The results obtained in this study are comparable with those obtained by Brown, J. C. (Brown, *et al.,* 1996). In his study, it was reported that at position 83, a mutation occurred (TCC-TTC) that resulted in the replacement of serine by

phenylalanine. It was also reported that at position 87, a mutation occurred (GAC – TAC) that resulted in the replacement of aspartic acid by tyrosine, similar to the results obtained by us. In the same study, double mutation was observed in strains resistant to both nalidixic acid and ciprofloxacin (RR pattern). Here the mutations has occurred at both positions simultaneously, 83 and 87 with replacement of aspartic acid by tyrosine at position 87 and serine by phenylalanine at position 83. In another study conducted in Japan by Hirose, J (Hirose*, et al*., 2002). Identical results of double mutations at positions 83 and 87 were obtained with RR-pattern. In the RS pattern, either a mutation at position 87 or a mutation at position 83 were identified. In our study, for the RS-pattern, only one mutation was identified at position 87. The difference between the Japanese study and ours could be resolved by sequencing more specimens belonging to the RS–pattern.

The relationship between *Salmonella* serotypes and the mutation (s) carried in *gyrA* has not yet been fully studied. A study by Giraud *et al* suggested that mutations at Ser 83 and Asp 87 may not be equally distributed among different serovars (Giraud *et al*., 1999). In this study complete serotyping was not carried out but the strains were differentiated into five serogroups (A, B, C, D and E). It has been noticed that most RR and RS in clinical as well as food isolates were of serogroup C. Further studies should be carried out to completely serotype the strains into their serovars and correlate the prevalence of mutation (s).

Recommendations

It is apparent from this study as well as many others, the dangers of using antibiotics in general in animal feed and quinolones in particular. It is clear that quinolones are used to treat severe *Salmonella* infections. Evolution of resistance to this class of antibiotics is extremely dangerous and may contribute to the spread of multidrug resistance *Salmonella* among humans. In the United States, the FDA prohibits the use of quinolones in animals and animal feed. This explains the quinolone resistance in that country of 2.3% as compared to much higher rates in third world countries (59.2% in our study) and Latin America. The Ministry of Health in Palestine must take this issue in consideration and implement stringent rules to regulate the use of certain antibiotics in animal feed and to prohibit the use of others such as the quinolones.

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