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Sahar Haddad

Supervisor Certification

I certify that this thesis was prepared under my supervision in the College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

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We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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Date:

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4-Conclusion and Recommendation

4.1. Conclusions:

1- The incidence of gastrointestinal tract was high in children under 5 years old.
2- *Salmonella typhimurium* SM9 has a lethal effect on mice.
3- *Salmonella typhimurium* SM9 have large plasmid (Mega plasmid) that responsible for pathogenecity and antibiotic resistance.
4- *Salmonella typhimurium* SM9 causing mice liver dysfunctions through disturbing GOT and GPT production.
5- Ethidium bromide demonstrated a powerful activity as curing agent in eliminating of plasmid responsible for pathogenecity and antibiotic resistance gene in *Salmonella typhimurium* SM9.
6- *S. typhimurium* resist to eight antibiotics, ampicillin, tetracycline, amoxicillin, gentamycin, nalidixic acid, streptomycin, kanamycin, and cephalexin. Five of them (ampicillin, tetracycline, amoxicillin, gentamycin, kanamycin) are conferring by plasmid DNA, While the other three antibiotics (nalidixic acid, streptomycin, cephalexin) are conferred by chromosomal DNA.
7- Large plasmid of *Salmonella typhimurium* SM9 is conjugative or self transmissible plasmid.

4.2. Recommendations:

1. New generation of antimicrobial agents must be developed, to cope with the multi resistance patterns of *Salmonella typhimurium* and used for therapeutic agent.
2. Study of the virulence factors that have role in pathogenesis of *Salmonella typhimurium* for example: enterotoxin, LPS, etc.
3. Investigate the mode of action of different virulence factor *in vivo* and *iv vitro*.
4. Histological study on the effect of *Salmonella typhimurium* SM9 on tissues.
5. Vaccine production from *S. typhimurium* by genetic engineering method.

1.2. Literature Review

1.2.1. *Salmonella:*

Salmonella spp. is responsible for the vast majority of food-borne illnesses across the world. Although most often this bacteria cause self-limiting gastroenteritis, it can also cause life-threatening disease such as typhoid fever (Sallyers and Whitt, 1994).

Salmonella was first isolated by the American scientist Danyle Salmone at 1885, it considered a member of *Enterobacteriaceae*, that are pathogens caused infection to human, animal and insects (AL-Hayali , 1993; and Holt *et al*., 1994).

Salmonella was straight rods 0.7-1.5 × 2.5 mm, gram negative, usually motile by peritrichous flagella, facultative and aerobic. It was chemo-organotrophs, have both respiratory and fermentative type of metabolism, the optimal temperature for growth is 37ºC, producing H_2S ,acid and gas from D-Glucose, L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, trehalose, and D-xylose.

Salmonella was negative for oxidase, indol and voges-proskaure tests, which it was positive for catalase, methyl red and citrate tests. Usually it was producing lysine and ornithine decarboxylases, variable in arginine dihydrolase (Holt *et al*., 1994).

Molecular studies classify *Salmonella* into, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided in to seven phylogenetic groups, subspecies I, II, III, IV, V, VI, and VII, depending on the biochemical characteristic and similarity of DNA (Reeves *et al*., 1989) .On the other hand ecologists classify *Salmonella* to three species, *Salmonella choleraesuis (bongori), Salmonella typhi* and *Salmonella enterica* as indicated in table (1-1).

Table (1-1) Ecologic Classification of *Salmonella* (Reeves *et al*., 1989)

Species	Representative Serovar(s)	Reservoir (host preferences)
Salmonella choleraesuis (bongori)	Only one	Animals (swine)
Salmonella typhi	Only one	Humans
Salmonella enterica	*Paratyphi-A Schottmuelleri*	Humans
	Pullorum	Animals(towel)
	Dublin	Animals(cattle)
	Typhimurium Derby Entertidis	Humans and many Animals

Salmonella species have more than 2200 remaining serovars depending on somatic and flagellar antigens, more of these remaining serovrs considered pathogens for human and animal (Aabo *et al*., 1993).

Salmonella possess three major antigens: H (flagellar antigen), O (somatic antigen), and Vi antigen (possessed by only a few serovars). H antigen may occur in either or both of two forms, called phase 1 and phase 2. The organisms tend to change from one phase to the other, O antigens occur on the surface of the outer membrane and are determined by specific sugar sequences on the cell surface, Vi antigen is a superficial antigen overlying the O antigen; it is present in a few serovars, the most important being *S.typhi* (Cruickshank *et al*., 1975).Antigenic analysis of *Salmonella* by using specific antisera offers clinical and epidemiological advantages, determination of antigenic structure permits one to identify the organisms clinically and assign them to one of nine serogroups (A-I), each containing many serovars. H antigen also provides a useful

epidemiologic tool with which to determine the source of infection and its mode of spread (Brenner *et al*., 2005).

1.2.2. Salmonellosis:

Salmonellosis is the name for the acute lower gastrointestinal tract disease caused by infection with the bacteria *Salmonella enterica* which has over 2,000 serotypes for example *Salmonella typhimurium* (Bean *et al*., 1990).

Compared to clinical disease, infection of humans and animals is usually several folds more common even in the midst of outbreaks, the infection is usually contracted by ingestion of *Salmonella*-contaminated foodstuffs but can be acquired by inhalation or by exposure of mucous membranes, such as by splashing of contaminated urine or the use of a contaminated rectal thermometer (Giannella *et al*., 1973). The minimum oral dose required to infect healthy adults is typically 10^5 organisms but foodstuffs with buffers or high fat content or the consumption of oral antibiotics can reduce this threshold over a thousand fold. Salmonellosis is commonly manifested clinically in both man and animals by sudden onset, usually 12 to 72 hours after ingestion of the organism, of one or more signs of gastrointestinal infection including high fever, abdominal pain, diarrhea and sometimes vomiting, a clinical case usually lasts for several days but the clinical signs and disease course is variable, depending on host factors, bacterial strain and dose (Stephen *et al*., 1985).

Although usually restricted to the intestinal tract, invasive forms of the infection can reach the blood stream and can affect other body organs and cavities with severe consequences, sometimes without the presence of gastrointestinal signs, cases experiencing invasive disease can shed the organism in all body secretions, the disease is most severe and the risk of

death is the highest in the very young, the elderly, the debilitated and the immunocompromised (Amieva, 2005).

As noted above most infections are sub-clinical rather than clinical, meaning that because the clinical signs aren't present in the infected person or animal special laboratory tests are required to detect it, like other bacterial food-borne infections, clinical cases are more common during the summer than other seasons (Banatvala *et al*., 1999).

Salmonellosis is one of the most common forms of food borne illness with approx. It includes several syndromes according to Stone *et al*., (1994) and as following :

1- Gastroenteritis

2- Enteric fevers

3- Septicemia

4- Focal infections

5- An asymptomatic carrier state.

Specific serovars show a strong tendency 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, *Salmonella* was found in the gastrointestinal tracts of many species of animals, birds, reptiles, and humans, and also in the environment which may be contaminated by the feces of animals and people (Benenson, 1996).

1.2.3. *Salmonella typhimurium:*

Salmonella typhimurium is facultative intracellular pathogen that cause varity of infectious disease, the most common of such disease is gastroenteritis with bacterial multiplication in intestinal, submucosa and

diarrhea, caused by the inflammatory response and perhaps also by toxins, *Salmonella typhimurium* is a broad-host-rang pathogens (Helmuth *et al* ., 1985; and Sallyers and Whitt, 1994), it is an emerging multi-drug resistant strain that was first reported in the United Kingdom in 1984; it is multiply-antibiotic resistant; the resistance is stable, i.e., doesn't need the selective pressure of antibiotic usage it to be maintained; the epidemic strains of *Salmonella typhimurium* are commonly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (Low *et al*., 1996).

1.2.4. Epidemiology of *Salmonella typhimurium*:-

Infection cases with salmonellosis increased through the seventeenth and eighteenth century with increases the rate of infection in some western countries, *Salmonella* bacteria considered as the causative agent to food poisoning in the world, The out break of this disease led to cause epidemics that include the human and animal (Ikeda and Hirsh, 1985).

In India the number infection between (1979-1982) about 300.000 cases and number of death about 1000 person in year, the rate death at age (1-4) year is 22%, at (15-45) year the rate death is 55% and at age more than 55 year 23% (Bhatia and Inchhpujam, 1994).

Diary and different animal products play important role in distributing infection cases with *Salmonella*, for example in Gorjeya – USA there is outbreak of infection with *Salmonella* as result of used ice cream products (Mahon *et al*., 1999).

Through epidemiological study on human *Salmonella* between 1991-1996 in Taiwan, it was found that the more serotype commonly retained to *Salmonella typhimurium* (Chen *et al*., 1999).

Salmonella cause 1 to 5% of gastroenteritis in developing countries, representing the most frequently isolated gram-negative bacteria in African countries (Falchi *et al*., 2003).

In Egypt since (1997-2000) that are found, *S. typhimurium* caused infectious diseases in the first six weeks of chicks life, also found that *S. typhimurium* continues to be a major or cause of food borne infections, with poultry being a major source of this infection in industrialized countries (Aly, 2003).

The occurrence of *Salmonella typhimurium* in chicken meet, giblets, pork meat beef , chicken meat balls, fish balls, shrimp balls, park balls, beef balls and sausages sold in open markets supermarkets and retailers shops ranges from 10 to 86% (Swetwiwathana *et al*., 1994).

In Iraq through epidemiology studies for *Salmonella* diseases between 1989-1985 found that infection rat every year (7.5) cases for 100.000 person .But Al-Hayali, (1993) considered that the rat more than 100 once than the recommended cases, and the reason is un recommended and loss cases for many reasons, so the pathogenic cases for typhoid and non-typhoid recommended for one year about 1286 cases.

1.2.5. Virulence Factors of *Salmonella*:

The virulence factors of *Salmonella* is the bacterial product that is necessary for causing disease, loss of any of the virulence factor led to reduce or loss pathogenecity, there are many of virulence factors that are play important role of different stage of infection and some of it similar to the virulence factor of *Escherichia coli* and *Virbrio cholera* (Finlay and Falkow, 1988). Among these virulence factors are:

1.2.5.1. Toxins:

Salmonella produce three types of toxins:

A- Enterotoxine:-

This toxin play important role in bacterial pathogeneses, it have molecular weight rang between 9×10^4 to 11×10^4 Dalton and the gene encoding this toxin is found to be chromosomally located (Sandefur and Peterson, 1976; and D' Aoust, 1989).

It has appeared similar characteristics to both heat stable –ST and heat labil –LT toxin for *E.coli*. This toxin is protein that is found in the cell wall or at the outer membrane, and it cannot be isolated from anther cell components (Koupl and Deibel, 1975).

Further more studies mention that this toxin is responsible for the diarrhea cases of the *Salmonella* food poisoning.

B- Cytotoxin:-

This type of toxin are found at the outer membrane of *Salmonella*, it is not- lipopolysaccharide its function is depression of protein synthesis in eukaryotic cell, this toxin is particularly destroyed by heat (Finlay and Falkow, 1988).

C- Endotoxin-(LPS):-

The cellenvelope of *Salmonella* contains a complex lipopolysaccharide (LPS) structure that is liberated on lysis of the cell; the lipopolysaccharide moiety may function as an endotoxin, and is important in investigating virulence of the organisms, this endotoxin complex consists of three components, an outer O-polysaccharide coat, a middle portion (the R core), and an inner lipid A coat (Galan, 1996).

Lipopolysaccharide structure is important for several reasons; first, the nature of the repeating sugar units in the outer O-polysaccharide chains is responsible for O antigen specificity; it may also help determine the

virulence of the organism, *Salmonella* lacking the complete sequence of O-sugar repeat units are called "rough" due to the rough appearance of the colonies; they are usually a virulent or less virulent than the smooth strains which possess a full complement of O-sugar repeat units (Internet 2). Second, antibodies directed against the R core (common enterobacterial antigen) may protect against infection by a wide variety of Gram-negative bacteria sharing a common core structure or may moderate their lethal effects; finally, the endotoxin component of the cell wall may play an important role in the pathogenesis of many clinical manifestations of Gram-negative infections, endotoxins evoke fever, activate the serum complement, depress myocardial function, and alter lymphocyte function (Internet 1).

1.2.5.2. Pili:

As a critical first step in the infection process, microorganism must come as close as possible to host mucosal surfaces and maintain this proximity by attaching to the host cell (adherence), the adhesive properties in the *Enterobacteiaceae* are generally mediated by different types of pili, these structures consist of polymeric globular protein subunit (pilin) molecular mass of 15 to 26 KDa (Ofek and Doly, 1994).

Pili are demonstrated manly on the basis of their ability to agglutinated erythrocytes of different animal species. Depending on weather the reaction is inhibited by D-mannose, the adhesions are designated as mannose-sensitive (MSHA) or mannose-resistant hemagglutinins (MRHA) according to Ottow, (1975).

A-Type 1 pili (MSHA):

This type of pili agglutinated guinea pig erythrocytes; the adhesion protein in this pilus type is located on the fimbrial shaft and is capable of binding to mannose-containing trisaccharides of the host glycoprotein (Babu *et al*., 1986).

The relevance of these pili to bacterial virulence is though to arise mainly from binding of bacteria to mucosal or to epithelial cells of the intestinal tracts (Venegas *et al*., 1995)

B- Type 3 pili (MRHA):

This factor considered to be secreting units that give some type of *Salmonella* ability to agglutinate RBC, it was found in *Salmonella typhimurium* and *Salmonella enteridis* that are found in digestive tract (Jones *et al*., 1982).

Unlike other fimberia, type 3 pili agglutinate only erythrocytes that have been treated with tannin, studies demonstrated that type 3 pili occur in many enteric genera, but they are not identical in all genera of enterobacteria (Old and Adegbola , 1985).

1.2.5.3. Virulence Factors of Invasion:

The entry of bacterial cell into eukaryotes cell is necessary step for bacterial virulence, it is completed mechanism that need more protein and DNA synthesis, the surface of epithelial cell caused activation to bacterial surface protein that are necessary for invasion and pathogencity (Finlaye *et al*., 1989).

It was found that the genetic markers responsible for invasion the epithelial cell localize on the chromosomal DNA, chromosomal part that responsible for bacterial invasion are classified as follows (Norel *et al*., 1989):

1- Invasion region (Inv).
2- Surface presentation antigen (Spa).
3- Invasion association genes (Iag).
4- Hyper invasion locus (Hil).
5- PhoP-repressed gene (phop).
6- Oxygen regulated gene.

While Al-Zagg, (1994) showed that importance invasion of mucous membrane of the digestive system and considered it as important step in virulence of *Salmonella typhimurium*, and the strain that unable to invasive considered as non pathogenic, also he was found component on the surface of the epithelial cell sensitive to tyrosine and teroaminase that stimulate the protein synthesis have virulence activity.

1.2.5.4. Sidrophores:

The growth of bacteria in host tissue is limited not only by host defense mechanism but also its supply of a viable iron, which is an essential factor in bacterial growth, functioning mainly a redox catalyst in protein participating in oxygen and electron transport posses (Khimji and Miles, 1978).

Reduce bacterial ability to taking up irons led to reduce of *Salmonella* pathogencity (D'Aoust, 1989).

The supply of free iron available to bacteria in host milieu is extremely low, since this element is bound intracellular to protein such as hemoglobin, ferritin, hemosiderin, and myoglobin and extracellularly to high affinity iron-binding protein such as lactoferrin and transferring, the level of free, bioavailable iron (10^{-18} M) is several thousand folds too low for normal bacterial growth (Bullen *et al*., 1987).

Many bacteria attempt to their supply of iron in host by secreting high-affinity , low molecular weight iron chelators ,called sidrophores that are capable of competitively up taking iron bound to host proteins (Griffith *et al* ., 1988).

Salmonella are synthesizing two type of sidrophores: first is phenolate-type sidrophores which known of enterobactin (also called of enterocolin), is acyclic trimer of 2,3-dihydroxy-benzoyl-serine. Enterobactin synthesis is by chromosomal gene, which found in most of the enteric bacteria (Khimji and Miles, 1978).

The second type is hydroxamate-type sidrophores which known as aerobactin and it is found in most pathogenic bacteria (Pyne, 1988).

1.2.5.5. Other Factors:

A- Physical factors:

Temperature, pH, pressure and other environmental conditions affected the virulence of bacterial isolate so the mutant *Salmonella* for temperature and pH is not caused infection to the digestive system for vertebrate (D'Aoust, 1989).

B- Structural factors:

Component of cell wall are need for bacterial virulence that are important for interaction between it and host, then caused infection (Wookcock, 1984).

The ability of bacterial cell to resist hard condition inside macrophage through out production of superoxide dismutase and catalase. Also production of lipopolysaccharide that are causative agent to death 25.000-50.000cases in USA every year, and it is important for *S. typhimurium* colonization in the intestine of mice (D'Aoust, 1989; and Mayeux, 1997).

1.2.6. Pathogencity and Mechanism of Infection:

An essential feature of the pathogenicity of *Salmonella* is their ability to engage the host cell in a two-way biochemical interaction. This interaction leads to responses from both the bacteria and the host-cell. *Salmonella* responds to the presence of the host-cell by activating a specialized protein secretion system termed Type III or contact dependent that is encoded within a pathogenicity island located at centisome 63 of the *Salmonella* chromosome .The majority of non-typhoidal *Salmonella* enter the body when contaminated food is ingested as in figure (1-1) (Wallis and Galydv, 2000).

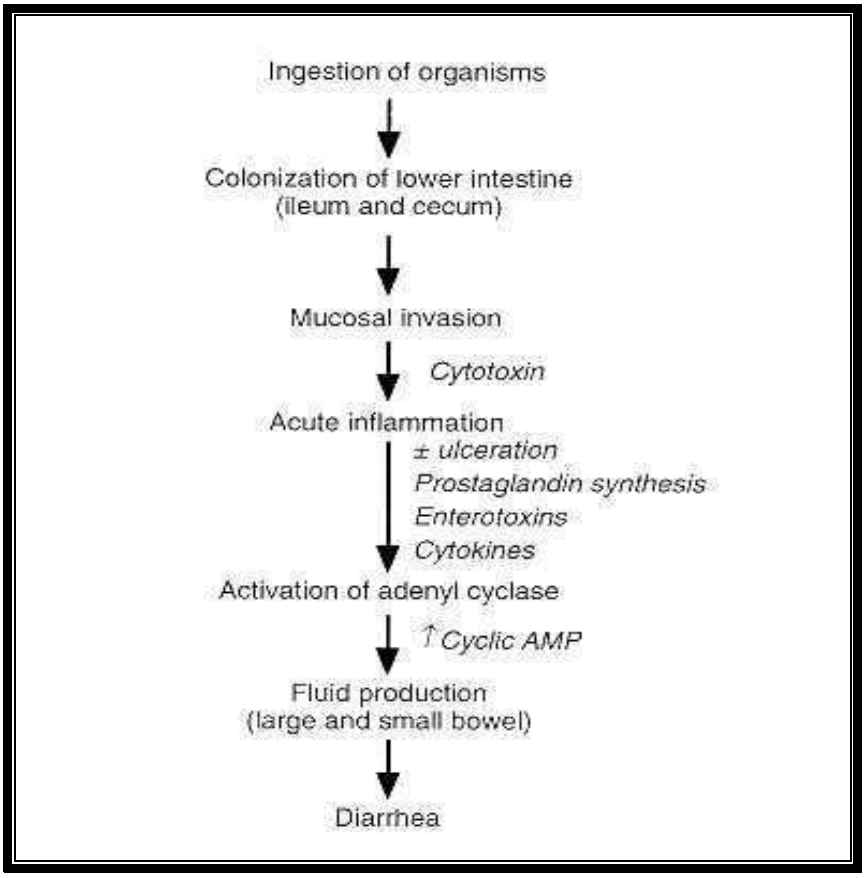

Figure (1-1) Scheme of the Pathogenesis of *Salmonella* (Wallis and Galydv, 2000)

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) the elaboration of toxins (Internet 2). After ingestion, the organism settles in the ileum and colon, attacks the intestinal epithelium, and proliferates within the epithelium and lymphoid follicles, the mechanism by which *Salmonella* invade the epithelium involves an initial binding to specific receptors on the epithelial cell surface followed by invasion (Internet 3). In non-phagocytic cells, *Salmonella* induces changes in the host-cell plasma membrane, and profound cytoskeletal rearrangements that closely resemble the membrane ruffles induced by a variety of influences, such as various hormones, growth factors, and the activation of cellular oncogenes, membrane ruffling is accompanied by macropinocytosis as in figure (1-2), ultimately leading to bacterial internalization (Wallis and Galydv, 2000).

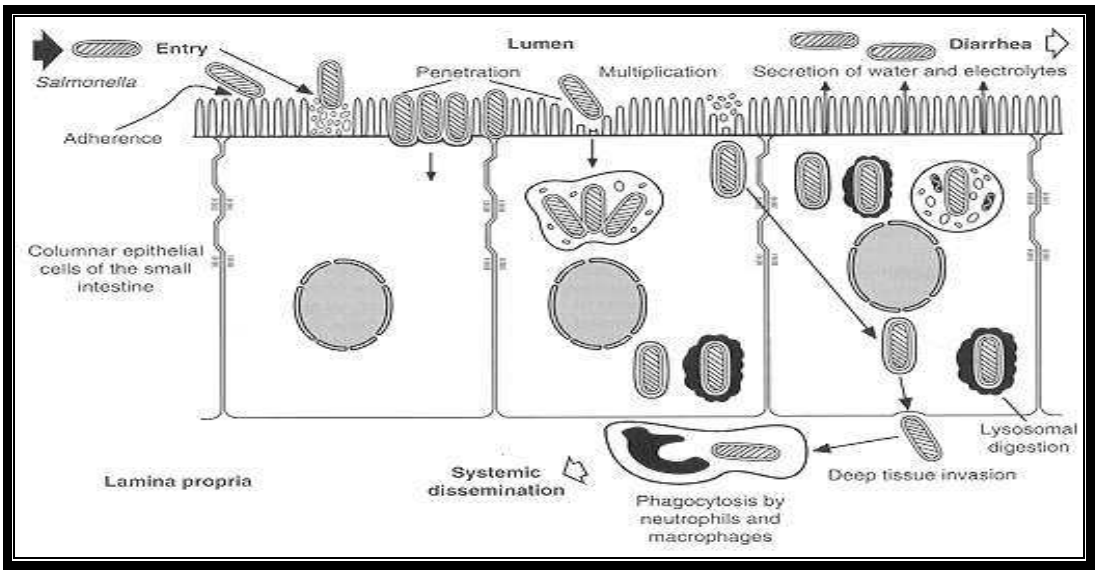

Figure (1-2) Invasion of intestinal mucosa by *Salmonella* (Wallis and Galydv, 2000).

After invading the epithelium, the organisms reproduce intracellularly and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation. The reticuloendothelial system limits and controls spread of the organism (Galan, 1996).

Following invasion of the intestine, most *Salmonella* cause an acute inflammatory response, which can cause ulceration; they may elaborate cytotoxins that inhibit protein synthesis. However, invasion of the mucosa causes the epithelial cells to activate various transcription factors, which ultimately result in the synthesis and release of pro-inflammatory cytokines (Wallis and Galydv, 2000).

1.2.7. *Salmonella typhimurium* and Antibiotics Resistant:

One of the major reasons for spreading the antibiotics resistance between bacterial population specially those that belong to the family *enterobacteriaceae* were due to most of the antibiotic resistance genes are chromosomal DNA , such as genes responsible for penicillin and cephalosporins résistance (Eggman *et al*., 1997), but these genes were carried also on self transmissible or mobilizable plasmids, and the transfer of such plasmids from one strain to another via conjugation or transformation play a vital role in multiple drug resistance (Livrelli *et al*., 1996 ; and Rice *et al*., 1996).

Multi resistant strain of *Salmonella typhimurium* comes from plasmids containing drug resistance genes that can be transferred among different species of enteric bacteria (Le Clerc, 1996).

Incorrect and long used of many antibiotics led to appear genes that not found, caused increase resistant of the bacterial strain (Chen *et al*., 1999).

Conjugation (direct *in vivo* gene transfer) is a convenient method of transferring drug resistant genetic determinants among intra- and intergeneric bacterial populations. This multi drug résistance led to antibiotic ineffectiveness against bacteria responsible for salmonellosis and other life threatening diseases (Rasool *et al*., 2003).

Poppe *et al*., (1998) found pathogenic *Salmonella typhimurium* for human and animal was multi-resistance antibiotics for ampicillin, chloramphenicol, streptomycin, and tetracycline.

Gazouli *et al*., (1998) found that two plasmids encoding for β-Lactamases in strains of *S. typhimurium*, one of them related to antibiotic resistance. Extended –spectrum β -Lactamases are encoded on conjugative plasmids, transposons, or integrons, these mobile genetic elements readily spread under selective antibiotic pressure (Jacoby and Sutton, 1991).

Multiple antibiotic resistances in *Salmonella enterica* serovar Typhimurium, an etiologic agent of food –borne enterocolitis in humans, are becoming a serious health problem (Low *et al*., 1996).

This is unique among *Salmonella* as most acquire antibiotic resistance genes by acquiring plasmids, which is extra-chromosomal DNA. To some experts, this suggests that this broad resistance will likely be retained by descendants of the epidemic strain even in the absence of the selective pressure of antibiotic use. With plasmid-mediated resistance, the conventional paradigm is that descendants of the resistant bacteria will discard the plasmid carrying a resistance gene against an antibiotic in the absence of exposure to that antibiotic, this loss is believed to occur because replication of the plasmid places that strain at a competitive disadvantage compared to non-plasmid carrying bacteria, with the resistance genes integrated into the chromosome, this mechanism of loss won't occur. Further, these genes are part of a "cassette" or integron, a

mechanism that enables easy exchange of genes between quite different, unrelated bacterial species. This means of bacterial genetic exchange was recognized only recently so the implications are not yet fully known. It is conceivable that this "cassette" could appear in other completely unrelated bacterial pathogens that are currently susceptible to these commonly used antibiotics (Threlfull *et al*., 1996).

1.2.8. Virulence Genes and Steps of Pathogenesis:

There was many attempts to drown genetic map for *Salmonella* bacteria detect virulence genes and know his position on the genetic map, first in 1965 when detect 133 genes only on the chromosome then there was attempts in 1966,1970,1972,1983, until Sanderson and Roth, (1988), drown the genetic map to *Salmonella typhimurium* and detect position of 680 gene on the map ; so it formed from 100 unit depending on the phage P22, this phage have ability to hold 45 Kb of DNA that represent about 1% from all size of chromosomal DNA of *Salmonella* .

The first step of pathogenesis depending on the genes localized in part size 4Kb from the chromosomal DNA that represent *Salmonella* pathogenesis region called *Salmonella* pathogenecity island 1 (SPI1), this region important to give *Salmonella* virulencity (Marcus *et al*., 2000; Hensel *et al*., 1999; and Shea *et al*., 1996).

While diagnoses invasion regulation of *Salmonella* to the epithelial cell in figure (1-3), good invasion of *Salmonella* to the epithelial cell need to produce secretion protein for invasion called sips , that work on the surface of epithelial cell and inside it, then change shape of cell membrane, at last caused mciropinocytosis, the sips proteins produce by secretion system called type III and it was stimulate by tach between bacteria and epithelial cell membrane (Brumell *et al*., 1999; and Guiney, 1997).

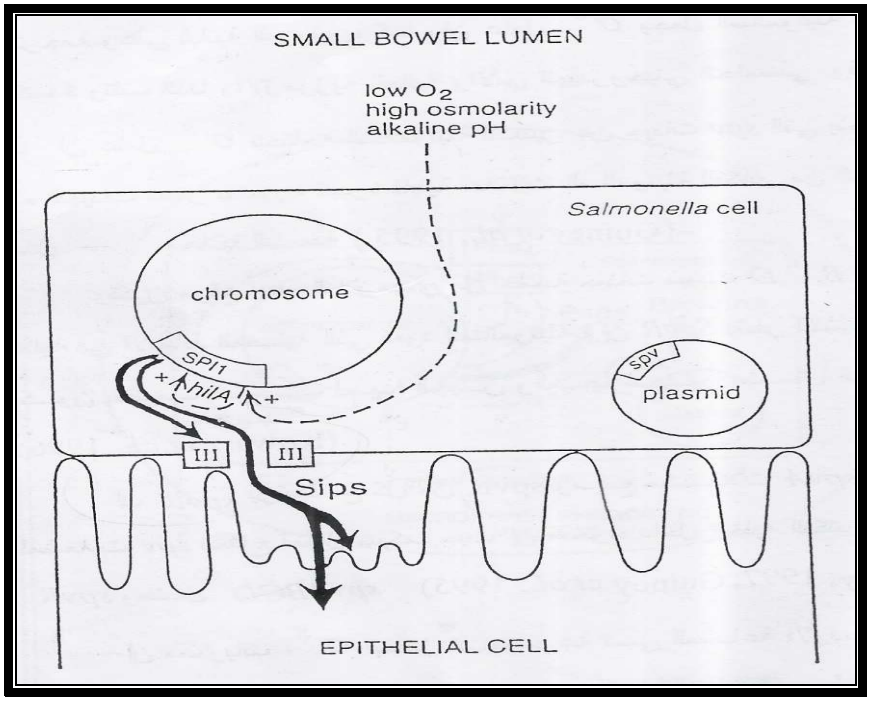

Figure (1-3) Regulation of invasion of *Salmonella* (Guiney, 1997).

Both Sips and type III was encoding through or by SPI1, expirations of this genes regulated by *hil A* gene product, and hiL A protein stimulate transcription many protein inside the pathogenicity island. The *hil A* gene localized in SPI1 region and it specialized regulate *Salmonella* invasion gene, gene expiration of *hil A* increased by sensitivity to the factor and condition that lead to expiration inversion genes, example of this factor: low level of O_2, high osmotic pressure and condition inside small bowel lumen (Jones and Falkow, 1996; and Galan, 1996).The virulence genes that localized on the plasmid in some type of *Salmonella* SPV did not have role in pathogenesis at first stage (Guiney, 1997).

Salmonella pathogenicity island 2 (SPI2) is locus, positioned at a centisome 31 of *Salmonella* chromosome. It encodes a type III secretion system (TTSS), which is involved in the systemic phase of pathogenesis in *Salmonella typhimurium*. One phenomenon mediated by SPI2 which is absorbed in epithelial cell is the formation of *Salmonella*–induced

filaments (SIFs), a novel tubular endocytic compartment which contains markers of late endosomes/lysosomes (Brown *et al*., 2003 and Geddes, 2003).

At the second stage of pathogenesis, bacteria engulfed by macrophage cell in intestine then distribution in macrophages and in systemic organs; inside macrophage *Salmonella* replicated in the membrane–bound lymphocyte vacuoles, bacteria need many genes to stay and growth in these conditions. There is two regulatory circular system that stimulate virulence inside macrophage, one of them Alternate sigma factor (RpoS) σ^s and the other PhoPQ that formed from two complex as in figure (1-4) (Brown and Elliott, 1997; and Coynanlt *et al*., 1996).

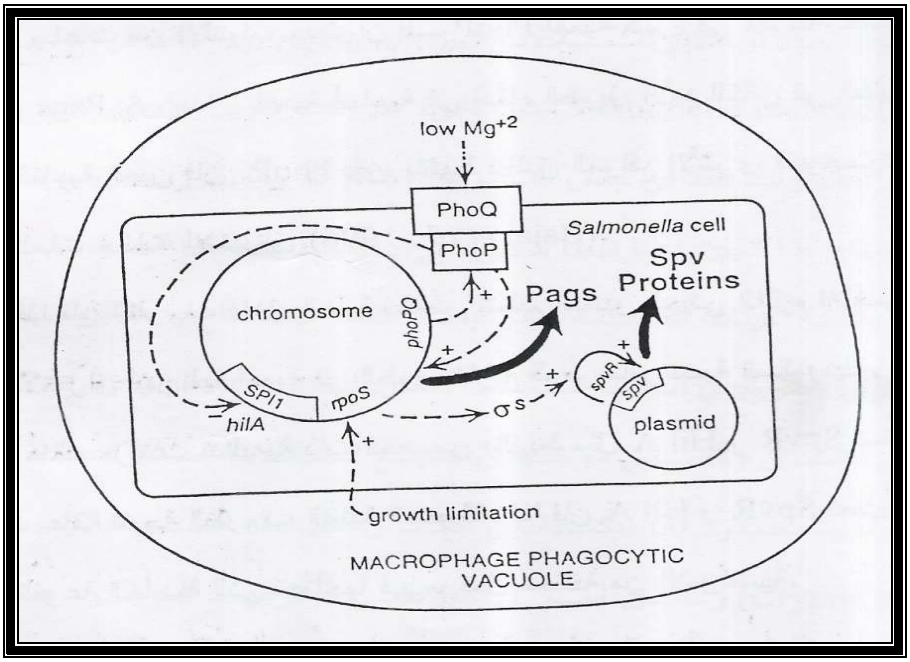

Figure (1-4) Regulation virulence gene in *Salmonella* responsible for infection macrophage cell (Brown and Elliott, 1997).

Alternate sigma factor σ^s regulates sensitivity to the finding condition. Growth markers affected by unstable condition that contain nutrient starvation, high osmotic pressure , low pH that σ^s stimulated by them , also expiration of gene *rpo S* regulated by completed mechanism work on transcription and expiration levels and on protein . σ^s factor make *Salmonella* more resistant to starvation oxidative factor ,DNA damage , high osmotic pressure and low pH, also *Salmonella* σ^s factor needed to *Salmonella* plasmid virulence (*s*pv) expiration that plasmid encoded to it (Guiney *et al*., 1995).

Another regulatory circular is PhoPQ that formed from two complex Phop and PhoQ control the expiration of virulence genes in *Salmonella* after phagocytosis (Guo *et al*., 1997)

Salmonella typhimurium virulence properties depend on the presence of large plasmid (65-100 Kb in size) to cause systemic infection (Jones *et al*., 1982; Terakado *et al*., 1983; Nakamura *et al*., 1985; and Gulig and Curtis, 1987).

Salmonella plasmid virulence (*s*pv) are heterogeneous in size (50-90 Kb) but all share a 7.8 kb region *s*pv, required for bacterial multiplication in the reticuloendothelial system, involved in the biosynthesis of fimbreiae and serum resistance, may play role in other stages of the infection process (Guiney *et al*., 1997; and Gulig *et al*., 1992).

Analysis has show that *Salmonella* plasmid virulence (*s*pv) harbors five gene spv^{RABCD}, this gene cluster, which have five open reading frames designated spv^R, spv^A, spv^B, spv^C and spv^D (Taira and Rhen, 1989; Krause *et al*., 1991; and Gulig *et al*., 1993).

The spv^R gene compine with chromosomally regulatory gene *ropS* (Alternate sigma factor σ^s), regulates exration of the spv^{ABCD} genes

(Taira *et al.,* 1991; Fang *et al*., 1992; Norel *et al*., 1992; and Gulig *et al*., 1993).

The spv^{R} protein is a positive regulator of spv^{ABCD} , regulation of the *spv* genes is dependents on the alternative sigma factor RopS , and the genes expressed within host cell (Fierer *et al*., 1993; Gulig *et al*., 1993; and Wilson *et al*., 1997)

*S*pvA protein (28 KDa) is found exclusively in the outer membrane, *s*pvB protein (66 KDa) that are found in two fractions and the largest fraction in cytoplasm, small amount of *S*pvB are found in the inner membrane, *S*pvC protein (28 KDa) is only detected in the cytoplasm, whereas *s*pvD (25KDa) is exported out side the cell (Although small amount are found in various cellular fraction). *s*pvR protein is (33KDa) (Roudier *et al*., 1992).

The function of the spv^{ABCD} gene products remains undetermined, but it has been shown that the presence of the virulence plasmid increases the growth of *Salmonella* in mice (Gulig and Doyle, 1993).

The *pef* (plasmid encoded fimbriae) locus contains four genes (pef^{BACDI}) named after the homology of their products with those of other fimbrial operons, the result of a search for genes able to complement a defection in cobalamin uptakes (Rioux *et al*., 1990).

The *traT, rck, rsk* genes also present in the several of *Salmonella typhimurium* virulence plasmid encode outer membrane proteins whose expression confers survival in macrophages cells (Heffernan *et al.*, 1992; and Rhen *et al*., 1992).

The large size of 90 kb virulence plasmid of *S. typhimurium* made it desirable to identify smaller specific regions of the plasmid that were involved in conferring virulence. Gulig and Curtiss used *in vivo* selection of virulence–conferring clones of the virulence plasmid to identify sequences associated with virulence in mice (Gulig and Curtiss III, 1988).

While Stein *et al*., (1996) was known the *Salmonella* virulence gene that responsible for formation filamentous component that have role in formation of bacterial colony on the surface of host cell, so he found that the mutant *Salmonella typhimurium* strains in *sifA* (*Salmonella* induced filaments) unable to stimulate formation of filamentous component. Also the *sifA* gene found only in *Salmonella* species and it encoded to protein at molecular weight 38 KDa.

1.2.9. Liver Enzyme GPT and GOT:

Enzymes are proteins that carry out most of the catalysis in the living organisms; they catalyze all the biochemical reactions by forming enzyme-substrate complex. Without assistance of enzymes most of the chemical reactions of metabolism would barely proceed at all (Atlas *et al*., 1995).

Transamination means the process of transferring an aminogroup from an amino acid to keto-acid. Enzymes which catalyze this type of reaction are named treansaminases and most important transaminase enzymes in diagnostics are Glutamic-Oxaloacetic-Transaminase (GOT) and Glutami-Pyruvic-Transaminase (GPT) According to Ricci and Federici, (1982).

The elimination of the toxic effects of drugs and chemical compounds by other different types of substances has been studied through the evaluation of liver enzymes level (Shamman *et al*., 1999; and Hassan, 2002).

A-Glutamate Oxaloacetate Transaminase (GOT):

This enzyme, also known as aspartate aminotransaminase, is widely distributed, with high concentrations in the heart, liver, skeletal muscle, kidney and erythrocytes, and damage to any of these tissues cause raised levels (Tietz *et al*., 1986).

GOT catalyzes the following reaction:

$$\textbf{L-Aspartate + } \alpha\textbf{-Oxoglutarate} \xrightarrow{GOT} \textbf{Oxaloacetate + L-Glutamate}$$

GOT exists in two isoenzyme forms, the mitochondrial form (M-GOT) and the cytosol form (S-GOT). Serum GOT levels in healthy subjects are low, but the levels are significantly elevated in a number of clinical conditions such as acute and chronic hepatitis, obstructive jaundice, carcinoma of the liver and myocardial infraction. Therefore, determination of serum GOT level has great clinical and diagnostic significance (Bergmeyer, 1974).

B-Glutamate Pyruvate Transaminase (GPT):

This enzyme, also known as alanine aminotransaminase, is found in higher concentration in liver and to a lesser extent in skeletal muscle, kidney and heart, the percentage of this enzyme differs from one person to another and from one tissue to another (Wong *et al*., 2000).

GPT catalyzes the following reaction:

$$\textbf{L-Alanine + } \alpha\textbf{- Oxoglutarate} \xrightarrow{GPT} \textbf{Pyruvate + L-Glutamate}$$

GPT is found only in the cytosol of cells (S-GPT), serum values more than 15-fold above the upper normal limit always indicate an acute

hepatocellular damage of viral, toxic or circulatory origin, in most types of liver diseases, GPT activity is higher than that of GOT, and GPT is more sensitive and specific in the detection of liver diseases (Pratt and Kaplan, 2001).

1.2.10. Plasmid Curing:

In nature, plasmid can be lost spontaneously from a very few cells, but the probability of this loss is extremely low, ranging from 10^{-5} to 10^{-7} (Molnar, 1988) however, the majorities of plasmid are extremely stable, and required the use of curing agents or other procedures that might increase the plasmid loss, and these from the basis of artificial plasmid elimination (Trevors, 1986; and Molnar, 1988).

Elimination of antibiotic resistance at high frequency is interested to assert extrachromosomal location of genetic determinants, and obtaining a plasmid-curing derivative will allow a direct comparison to be made between the plasmid-containing and plasmid cured cells.

As a result of earlier studies it is already known that acridin orange, ethidum bromide and sodium dodecyl sulfate (SDS) affect plasmid replication (Tomoeda *et al*., 1968).

Elevated temperature and thymine starvation also affect on plasmid replication (Groves, 1979; and Trevors, 1986).

Some antibiotic like rifampicin, chloramphenicol and mitomycin C also have a moderate effect on plasmid replication (Fenwick and Curtiss, 1973). Some tricyclic compounds like promethiazine and impramine were shown to have antiplasmid activity (Molnar *et al*., 1978; and Molnar, 1988).

1.2.11. Conjugation:

Bacterial conjugation is the transfer of genetic material between bacteria through cell-to-cell contact, it is a mechanism of horizontal gene transfer, as are transformation and transduction, although these mechanisms do not involve cell contact (Gale, 2005).

Bacterial conjugation is often incorrectly regarded as the bacterial equivalent of sexual reproduction or mating, it is not actually sexual, as it does not involve the fusion of gametes and the creation of a zygote, nor is there equal exchange of genetic material, it is merely the transfer of genetic information from a donor cell to a recipient. In order to perform conjugation, one of the bacteria, the donor, must play host to a conjugative or mobilizable genetic element, most often a conjugative plasmid, most conjugative plasmids have systems ensuring that the recipient cell does not already contain a similar element (Internet 4).

The genetic information transferred can be beneficial to the recipient, such as in conferring antibiotic resistance, or an enzyme that allows it to better digest its medium. However, these elements can also be viewed as genetic parasites on the bacterium, and conjugation as a mechanism evolved by the element to spread itself into new hosts (Gale, 2005).

The prototype for conjugative plasmids is the F-plasmid, also called the F-factor. The F-plasmid is an episome (a plasmid that can integrate itself into the bacterial chromosome by genetic recombination) of about 100 kb length (One kb is one thousand base pairs). It carries its own origin of replication, called *oriT*. There can only be one copy of the F-plasmid in a bacterium (which is then called *F-plus*), either free or integrated. Among other genetic information, the F-plasmid carries a *tra* and a *trb* locus,

which together are about 33 kb long and consist of about 40 genes (Sukupolvi *et a*., 1990).

Conjugation allows bacteria to increase their genetic diversity. Thus, an advantageous genetic trait present in a bacterium is capable of transfer to other bacteria. Without conjugation, the normal bacterial division process does not allow for the sharing of genetic information and, except for mutations that occur, does not allow for the development of genetic diversity (Internet 4).

Anderson and Lewis, (1965) founds that *Salmonella typhimurium* DT104 encoded all of their resistance genes on plasmids, unlike the chromosomally, which could be transferred to *Escherichia coli* via conjugation, but that the plasmid compositions varied among the isolates.

There are studies determined that there is a correlation in *Salmonella* isolates between the presence of several virulence plasmid loci and the F plasmid locus, *traD* (Boyd and Hartl, 1998). Based on this correlation, the researches hypothesized that the F plasmid and the virulence plasmid might be one and the same.

3. Results and Discussion:

3.1. Isolation of *Salmonella typhimurium*:

To isolate *Salmonella*, 100 stool samples were collected from children with ages under 5 years suffering from different diarrhea cases in Al-Kadhimia and Al-Elwia hospitals and Ibn Gaswan Hospital in Baghdad and Basra governorates respectively, stool samples were taken from both male and female patients using woody sticks, and transferred to test tubes containing sterile peptone water. After incubation, 10 ml of cultured stool samples in peptone water were transferred to conical flasks containing sterile tetra-thionate broth medium which an enrichment and selective medium for *Salmonella* isolates (Cruickshank *et al*., 1975). Flasks were then incubated at 37˚C for 24 hours, and then 100 µl aliquots of the cell suspension after incubation was transferred and spread on *Salmonella-Shigella* agar plates (SS agar) and incubated at 37˚C for 24 hours. SS agar medium is a high selective medium for *Salmonella* spp., which inhibits the growth of most coliform microorganism and permits the growth of species of *Salmonella* and *Shigella* from clinical specimens. The medium contains high bile salts concentration and sodium citrate, which inhibits all gram positive and many of gram negative bacteria. Lactose is the sole source of carbohydrate, and neutral red is used as an indicator for acid production. Sodium thiosulfate is a source of sulfur, and any bacteria that produce hydrogen sulfide gas (H_2S) are detected by a black precipitate formed with ferric citrate (Atlas *et al*., 1995).

3.2. Identification of *Salmonella typhimurium*:

3.2.1. Morphological Characterization:

Morphological characteristics of the isolates obtained after culturing on SS –agar were studied, results showed that 38 isolate out of the total (100 isolate) have the characteristics of *Salmonella* spp., colonies on SS agar were circular disk, 1-2 mm in diameter as that have gas bubbles and black spot as a result for production of H_2S, while on nutrient agar colonies were moderately large (2-3 mm in diameter), grey-white, moist, circular disk with a smooth convex surface and entire edge. Also on MacConkey's medium colonies were pale yellow or nearly color less, 1-3 mm in diameter, non lactose fermentation. These results are in agreement with Cruickshank *et al*., (1975).

Microscopically examination for these isolates showed that they were gram-negative, rod or bacilli these results are in agreement with Holt *et al*., (1994).

3.2.2. Biochemical tests:

Biochemical testes were achieved for the 38 isolates with growth and morphological characteristics closely related to *Salmonella* spp.

Results indicated in table (3-1) showed that only 10 isolates give negative reaction for urease, indole, and voges- proskaur tests respectively, while they were gave positive result to citrate utilization, methyl red, and glucose fermentation, all these isolates were motile, H_2S and gas production, so these 10 isolates were identified as *Salmonella typhimurium* according to Holt *et al*., (1994).

Table (3-1) Morphological and biochemical characteristics of locally isolates of *Salmonella typhimurium*

Characteristic		Isolates									
		SM1	**SM2**	**SM3**	**SM4**	**SM5**	**SM6**	**SM7**	**SM8**	**SM9**	**SM 10**
Cell shape		Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram Stain		—	—	—	—	—	—	—	—	—	—
Motility		+	+	+	+	+	+	+	+	+	+
TSI	H_2S	+	+	+	+	+	±	±	±	+	+
	gas	+	+	+	+	+	—	+	+	+	—
	K/A	±/+	–/+	+/+	–/+	+/+	–/+	+/+	+/+	+/+	+/+
Indol		—	—	—	—	—	—	—	—	—	—
Methyl red		+	+	+	+	+	+	+	+	+	+
Vogas-preskaur		—	—	—	—	—	—	—	—	—	—
Citrat Utilization		+	+	±	+	±	+	+	±	+	+
Urease Test		—	—	—	—	—	—	—	—	—	—
Glucose Fermentation		+	+	+	+	+	+	+	+	+	+

Where :: (+): positive control ; (-): negative control ; (K): alkaline ; (A): acidic (±): variable ; (TSI): Triple Sugar Iron

Moreover, identification of these 10 isolates as *Salmonella typhimurium* were also confirmed by using Api system (Api 20E) as shown in figure (3-1), the findings obtained by the convention biochemical test, these isolates were able to gave positive results to ornithine decarbxylase, citrate utilization , hydrogen sulphide, gelatin liquefaction, they are glucose, arabinose, sorbitol, rhamnose , melibiose , nositol, manitol fermentors. While they gave negative results to Beta-galactosidase, arginine dihydrolase, lysine decarboxylase, urease , tryptophane deaminase, indole , voges-proskauer, amygdalin fermentation, sucrose fermentation. These results were also in agreement with those mentioned by Holt *et al*., (1994). Furthermore identification of *Salmonella typhimurium* isolates was confirmed by using antisera grouping test, which was done according to the type of antigen. The identification of serotypes depends on detection of O (somatic) and H (flagaellar) antigens by means of agglutination test with specific antisera.

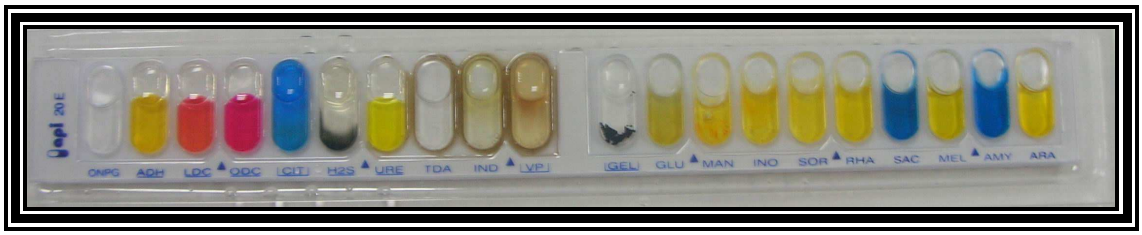

Figure (3-1) Api 20E system for identification of locally isolated *Salmonella typhimurium*

3.3. Selection of Virulent Isolate of *Salmonella typhimurium*:

In order to select the most virulent isolate of *Salmonella typhimurium*, each isolate was injected in mice intrapretonially with 0.1 ml of *S.typhimurium* suspension (Optical Density of cell suspension for each isolate was ranged from 0.43 to 0.47), then the time for death of animals after injection with each isolate was measured.

Result indicated in table (3-2) showed that *S. typhimurium* SM9 was the most virulent isolate because it causes death of animals after 19 hours, while other isolates causes death of animals after 24 to 96 hours. According to these results *Salmonella typhimurium* SM9 was selected to be the most virulent isolate and it was used in the next experiments of this study.

Table (3-2) Survival time of mice after injected with local isolates *Salmonella typhimurium**

Isolate	Optical Density (620 nm)	No. of injected cells (cell/ml)	Time of death (hour)
SM 1	0.43	10^8	96
SM 2	0.42	10^8	24
SM 3	0.47	10^8	48
SM 4	0.46	10^8	24
SM5	0.43	10^8	72
SM 6	0.47	10^8	–
SM 7	0.45	10^8	–
SM 8	0.47	10^8	72
SM 9	0.42	10^8	19
SM 10	0.46	10^8	–

(–): Still alive; (*): Each result in table represent a mean of duplicate.

Death of animals after infection with different isolates of *S. typhimurium* during different time intervals may be due to the infection of mice with typhoid-like disease **(**Helmuth *et al*., 1985**)**.

It was well known that, *Salmonella* was a pathogen causes a variety of infectious diseases. The most common of such diseases is gastroenteritis, with bacterial multiplication in intestinal submucosa and diarrhea, caused by the inflammatory response and perhaps also by toxin. In specific hosts, adapted *Salmonella* produce systemic diseases such as typhoid and paratyphoid fevers, it can inter the blood stream and cause septicemia, which is often lethal (Sallyers and Whitt, 1994).

S. typhimurium have many of virulence factors that responsible for pathogenicity are often encoded by plasmid, so the plasmid borne virulence genes that caused infection and led to death of mice (Boyd and Hartl, 1998).

3.4. Antibiotic Sensitivity:

The standard disk diffusion method was used to determine the sensitivity of locally isolated *S. typhimurium* SM9 to different antibiotics.

Results indicated in table (3-3) showed that *S. typhimurium* SM9 was resist to different antibiotics varied according to the type of antibiotic, however SM9 was resisted to ampicillin, tetracycline, amoxicillin, gentamycin, nalidixic acid, streptomycin, cephalexin, and kanamycin, while it was sensitive to tobramycin, rifampicin, carbencilin, trimethoprim and clindamycin.

Table (3-3) Antibiotic Sensitivity of *Salmonella typhimurium* SM9 to different antibiotics.

Antibiotic	Symbol	Sensitivity
Ampicillin	Am	R
Tetracycline	Tc	R
Amoxicillin	Ax	R
Gentamycin	Gm	R
Nalidxic acid	Nal	R
Chloramphenicol	C	Int
Streptmycin	S	R
Trimethoprim	Tp	S
Rifampicin	Rif	S
Tobramycin	Top	S
Cephalexin	Cfx	R
Clindamycin	CD2	S
Kanamycin	K	R
Carbencillin	Py	S

(R) : resistant ; (S) : sensitive ; (Int) : intermediate

From these results, it noticed that *S. typhimurium* SM9 was resist to eight antibiotics, while it was sensitive to five antibiotics. Resistant to antibiotics may be come from the antibiotic resistance genes; some of these genes may be located on chromosomal DNA, while the others may be located on plasmid DNA (Jawetz *et al*., 1998).

These results were in agreement with Low *et al*., (1996) who found that 65% of all *Salmonella* isolates have been identified as *Salmonella typhimurium* were resistant to ampicillin, cholramphenicol, streptomycin and tetracycline. These phenotypes of *Salmonella typhimurium* come from chromosomal and plasmids containing drug resistance genes, well known in bacteria and can be transferred among different species of enteric bacteria. Further more, a relatively high frequency of hyper mutable strains among pathogenic *Salmonella* spp. could be found (Le Clerc, 1996).

Incorrect and long used of many antibiotics led to appear genes that not found previously, causes increase in resistance of the bacterial strain (Chen *et al.*, 1999).

The multidrug resistance of bacteria to several antibiotics could be due to the permeability of the outer membrane, which might prevent the entry of antibiotic into cell, or due to certain mutations that occur as a result of over use and misuse of antibiotics, but arise spontaneously are generally resistant to only one antibiotic (Malkawi and Yousef, 1996; AL- Shallchi, 1999; and Rasool *et al*., 2003).

In addition to mutations, R plasmids offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact. Conjugation (direct *in vivo* gene transfer) is a convenient method of transferring drug resistant genetic determinants among intra- and intergreneric bacterial populations (Rasool *et al*., 2003; and Amyes *et al*., 1989).

This multidrug résistance led to antibiotic ineffectiveness against bacteria responsible for salmonellosis and other life threatening diseases (White *et al*., 2002).

Gazouli *et al*., (1998) found plasmid encoding for β-Lactamases in strains of *S. typhimurium*, which wide spread of ampicillin resistance is attributed to degradation of the antibiotic by β-Lactamases.

The reason of resistance *S. typhimurium* to nalidixic acid may be related to mutate in ser-83 or Asp 82 to phe in Gyr A protein in *gyrA* gene (Heurtin *et al*., 1999; and Ruiz *et al*., 1997). While resistance to trimethporim due to production of enzyme DHFR- Dihdrofolate reductase, coding by plasmid DNA distribution between *Enterobacterasea* (Young and Ltillyear, 1994; and Ringertz *et al*., 1990).

Sensitivity to rifampicen can be explanation by rare used it as antibiotic because it considered as mutant agent (Trevors, 1986).

3.5. Haemolysin Production:

Salmonella typhimruimn SM9 was tested for haemolysin production on blood agar medium as in (2.2.11).

Results shown that this isolate is not able to produce haemolysin because there were not any haemolysis zones around the colonies of *S. typhimurium* SM9.

These results agreed with those obtained by Mutloob, (2000) who found that different isolates of *S. typhimurium* were unable to produce haemolysin.

Haemolysin is protein able to haemolyse the RBC and have different molecular weight from bacteria to another, it consider as additional virulence factor for bacteria (Nassif and Sansonetti, 1986; and Gadeberg and Biorn, 1986).

3.6. Sidrophore Productions:

Salmonella typhimurium was tested for sidrophore production on sidrophore production medium as in (2.2.12).

Results shown that *S. typhimurium* SM9 was able to growing on selective medium for production of sidrophore. These results agreed with those results obtained by Matloob, (2000), who found that different isolates of *S. typhimurium* were sidrophore producers.

Sidrophore have high affinity to iron with low molecular weight, as a virulence factor for *S. typhimurium*. Sidrophore are important for the bacterial cells to chelate iron compounds from the host tissue; and then take up it from the low affinity complex that facilitated transport it inside cell by specific receptor (Johnson, 1991).

These results are agreement with Carramin~nana *et al.*, (1997); who found that 80% of *Salmonella* isolates from patient specimen were positive for the sidrophore test.

3.7. Assay of Mice Liver GOT and GPT Enzymes:

In this study, effect of the injected *S. typhimurium* SM9 on mice GOT (glutamate oxaloacetate transaminase) and GPT (glutamate pyruvate transaminase) levels have been investigated. Results in table (3-4) showed that there is significant increase ($P<0.05$) in GPT level after injection with 10^5 cell/ml of *S. typhimurium* SM9, in comparison with the negative control. The activity of GPT was increased significantly ($p<0.05$) from 199.3 U/L for the positive control to 375.1 U/L for the injected mice.

Significant increase in mice GPT may be due to the toxic effect of different compounds produced by *S. typhimurium* SM9 on the function of mice liver, since the liver is the major organ in which the metabolism and detoxification of different compound was occur as it was mentioned by Mathur and Dive, (1981).

On the other hand, there is significant increase ($P<0.05$) in mice GOT level after injection with 10^5 cell/ml of *S. typhimurium* SM9, in comparison with the negative control. The activity of GOT was increased significantly ($p<0.05$) from 68.0 U/L for the positive control to 90.81 U/L for the infected mice.

These results were in agreement with Bergmeyer, (1974) who referred that the levels of GOT and GPT increased in a number of clinical conditions such as acute and chronic hepatitis, obstructive jaundice, carcinoma of the liver and myocardial infraction.

Table (3- 4) Assay of mice liver GPT and GOT before and after injected with 10^5 cell/ml of *S. typhimurium* SM9.

Treatment	GPT m±SE (U/L)	GOT m±SE (U/L)
Injected with PBS (Positive Control)	A 199.32±26.16	A 68.00±0.94
Injection with *Salmonella typhimurium* SM9	B 375.19±12.54	B 90.81±2.07

Differences letters represents significant differences ($P<0.05$).

In general the increase of GPT and GOT may be also due to the cytotoxic effect of *Salmonella typhimurium* SM9 on liver cells and this leads to increase the permeability of liver cell membrane or to cause

damages in liver tissue and thus causing the release of high levels of these enzymes into blood stream (Bonnefoi *et al*., 1989; D'Mello *et al*., 1999; AL-Obaidy, 2001).

Also there are chemotherapeutic drugs like tamoxifen that cause an increase in serum levels from GPT and GOT in animal treated with it, this may be due to the cytotoxic effects on the liver cells cause damages of liver tissue and increases GPT and GOT levels (D' Mello *et al* ., 1999).

Berry and Smythe, (1964) reported an increase of some liver enzyme activity in animals injected with *Salmonella typhimurium*, this increase level of activity of enzymes, with decreases of other liver enzyme, led to animals succumbed and death. So injected animals may be related to inability of these animals to utilize glyconeogenic intermediates .Snyder *et al*., (1971) associated the increased activity of pyrovate kinase (PK) in injected animals with the depletion of liver glycogen.

3.8. Plasmid Profile:

Plasmid profile of *S. typhimurium* SM9 was studied in order to know the role of plasmid DNA in the virulence of this isolate. Plasmid DNA was extracted according to the modified alkaline lysis method described by Birnboim and Doly described by Larvery *et al*., (1997).

Result in figure (3-2) showed that the locally isolated *S. typhimurium* SM9 have a mega plasmid after electrophoresis on agarose gel. The

position of the mega plasmid DNA band is over the position of the chromosomal DNA.

This result was agreed with those results referred that most *S. typhimurium* isolates (88%) carry a plasmid of 60 kb (Helmuth *et al*., 1985). This plasmid has been given a variety of names pSLT, MP10, pRQ28, pSTV, the cryptic plasmid, and the virulence plasmid (Helmuth *et al*., 1985; Ou *et al*., 1993; and Sanderson**,** 1996).

The most apparent consequence of virulence plasmid carried is to enhance the growth rate of the bacterium during systemic phase of disease (Gulig and Doyle, 1993).

Alkaline lysis method is a suitable method for extraction of plasmid DNA. Lysozyme treatment is an important step in cell lysis, while proteinase k has been useful in the preparation of lysates and probably aids in breaking up DNA-protein-membrane complexes after cell lysis (Clewell *et al*., 1974).

During these steps, 5M potassium acetate was added to the lysate to allow preciption of chromosomal DNA, high molecular weight RNA-protein-membrane complexes (Maniatis *et al*., 1982).

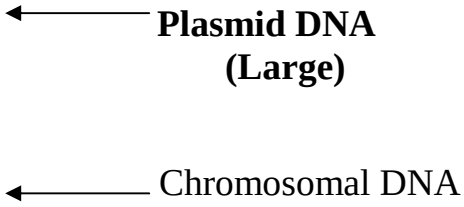

Figure (3-2) Plasmid Profile of *Salmonella typhimurium* SM9 on agarose concentration 0.7 (W/V) and voltage 70 V for 6 hrs

3.9. Plasmid Curing:

In order to know the relationship between the plasmid profile and virulence of *S. typhimurium* SM9. Many attempts were made in order to cure *S. typhimuirum* SM9 using ethidium bromide as intercalating agent according to procedure described by Trevors, (1986).

The mode of action of ethedium bromide in curing of plasmid DNA is the inactivation of replication of plasmid DNA during cell division without any effect on the chromosomal DNA replication, which leads to the presence of plasmid-less cell in the next generations. Furthermore ethidium bromide was a good agent in curing of plasmid DNA, if it compared with physical and other chemical agents (Hohn and Korn, 1969).

First of all, fresh culture of *S. typhimurium* SM9 was used to inoculate luria-bertani broth medium containing gradual concentrations of ethidium bromide, and incubated for 24 hrs at 37˚C to determine the sub-lethal concentration.

Results indicated in table (3-5) showed no growth of SM9 was noticed in the sub-lethal concentration of ethidium bromide (1000 µg/ml) allows the growth of *S. typhimurium* SM9.

From the culture containing the sub-lethal concentration of ethidium bromide, serial dilutions were taken and spread on brain heart infusion agar plates, and incubated at 37˚C for 24 hrs. Then one hundred of the resultant colonies were replica plated on brain heart infusion agar plates containing different antibiotics (ampicillin, tetracycline, gentamycin, streptomycin, amoxicillin, cephalexin, nalidixic acid, and kanamycine).

Table (3-5) Effect of Ethidium Bromide on the growth of *Salmonella typhimurium* SM9 after growing in luria-bertani broth at 37˚C for 24 hrs.

Ethidium bromide concentration (µg/ml)	Growth
0	+++
20	++
50	++
100	++
200	+
300	+
400	+
600	+
800	+
1000	±
1600	−

Where:

(–): no growth.

(±): slight growth.

(+): moderate growth.

(++): good growth.

(+++): very good growth.

Result showed that most of these colonies were still resist to these antibiotics, while few colonies fail to grow on plates containing ampicillin, tetracycline, gentamycin, amoxicillin, kanamycine. This may due to plasmid curing caused by the effect of ethidium bromide as a curing agent and as it was mentioned by Bouanchaud *et al*., (1969).

From these results it can be concluded that resistance to ampicillin, tetracycline, gentamycin, amoxicillin, and kanamycine were located on plasmid DNA (plasmid encoded phenotypes), while cephalexin, nalidixic acid, and streptomycin phenotypes were chromosomally located.

To confirm this result, cells that suspected to be cured was taken and grown in luria broth for 24 hrs at 37˚C with shaking (150 rpm), and then plasmid profile was examined by extraction using alkaline lysis procedure.

Results in figure (3-3) showed that cured cells were lost its plasmid DNA on agarose gel, which mean the wild type of the *S. typhimurium* SM9 was herber of a mega plasmid conferring the resistance for five different antibiotics also to other genes and phenotypes.

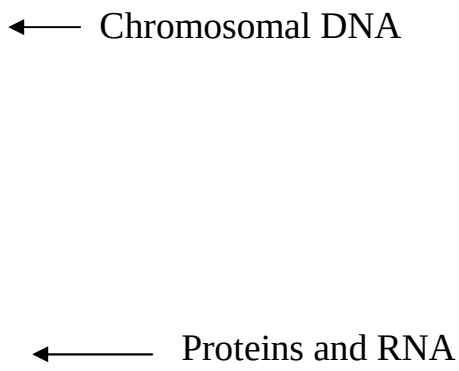

Figure (3-3) Gel electrophoresis of total DNA extracted from *S. typhimurium* after curing by ethidium bromide.

Agarose concentration 0.7 % (W/V).

Voltages 70 V.

Time 2 hours.

3.10. Assay of Mice Liver Enzyme GOT and GPT After Curing:

Effect of the injected cured cells of *S. typhimurium* SM9 on liver GOT and GPT levels has been investigated. Results in table (3-6) showed non significant differences in GPT level when injected with 10^5 cell/ml of cured cells of *S. typhimurium* SM9 (242.80 U/L), in comparison with the positive control (199.32 U/L).

On the other hand, there is non significant difference in GOT level when injected with 10^5 cell/ml of cured *S. typhimurium* SM9 (64.00 U/L), in comparison with the positive control (68.00 U/L).

Table (3-6) GPT and GOT levels before and after injection with 10^5 cell/ml of cured *S. typhimurium* SM9.

Treatment	GPT m±SE (U/L)	GOT m±SE (U/L)
Injection with PBS (Positive control)	A 199.32±26.16	A 68.00±0.94
Injection with cured *Salmonella typhimurium* SM9	A 242.80±40.59	A 64.00±9.06

Same letters represents non significant differences.

These results came in according to Gulig and Doyel, (1993) whose founds that *Salmonella* plasmid virulence (spv) genes enable the bacterium to infect the spleen and liver by increasing the rate of bacterial replication within host cells. But plasmid-cured strains are able to colonize and persist in spleen and liver, but bacterial growth is controlled by host defenses and infection dose not developed.

3.11. Conjugation:

It is well known that most of the mega plasmid is conjugative or self transmissible; conjugation is a good method to study the characteristic of the virulence plasmid and its role in pathogensity. It was found that 50% of *Salmonella* isolates contain an F plasmid; it was found that there is a correlation in *Salmonella* isolates between virulence and F plasmid (Boyd and Hartl, 1997).

According to these information's, conjugation was achieved between *S. typhimurium* SM9 (donor), which has the mega plasmid as it was described previously in (3.8) and *E. coli* MM 294 (recipient).

Transconjugants were selected according to the phenotypes (antibiotic resistance) present in both of the donor (resistance to ampicillin) and the recipient (resistance to rifampicin).

Using MacConky agar plates. This medium allows the growth of transconjugants and do not permit the growth each of the recipient and donor cells.

Results showed that transconjugants were able to grow on the MacConky agar plates containing the (ampicillin and rifampicin). These results confirm the transferring the conjugative plasmid (mega plasmid) of the locally isolated *S. typhimurium* SM9 to *E. coli* MM 294 frequency of conjugation was determined, and it was found to be 10^{-7} cell/ml (Nasir *et al*., 2004).

To confirm conjugation and the transfer of the conjugative plasmid from *S. typhimurium* SM9 to *E. coli* MM 294, plasmid profile of transconjugates was studied by extraction of total DNA according to

alkaline lysis method, and then examined after electrophoresis on agarose gel.

Result indicated in figure (3-4) showed that transconjugants *E.coli* MM 294 that able to grow on selective media, have two distinct bands after electrophoresis on agarose gel for 6 hours. The first band represents the conjugative plasmid (large plasmid) of *Salmonella typhimurium* SM9, while the second band represents chromosomal DNA of *E. coli* MM294.

As a result this plasmid is conjugative plasmid, it have characteristic of resistance to antibiotics (Ampicilin, Amoxicillin, gentamycin, Kanamycin) and it is able to expression in *E.coli* cell.

In *Salmonella* isolates there is a correlation between the presence of several virulence plasmid loci and the F plasmid locus, *traD* (Boyd and Hartl, 1998). Based on this correlation, they hypothesized that the F plasmid and the virulence plasmid might be one and the same. This virulence plasmid might be self-transmissible (Jones *et al.*, 1982; Ou *et al*., 1993; and Sanderson *et al*., 1983).

The demonstration that the virulence plasmid of *S. typhimurium* is self-transmissible provides an example of horizontal gene transfer. The fact that this genetic exchange involves virulence factors has implications for the evolution of enteric pathogens such as *Salmonella*. The horizontal transfer of genetic material by conjugation is likely to increase the evolutionary rate at which pathogens can test new virulence gene combinations.

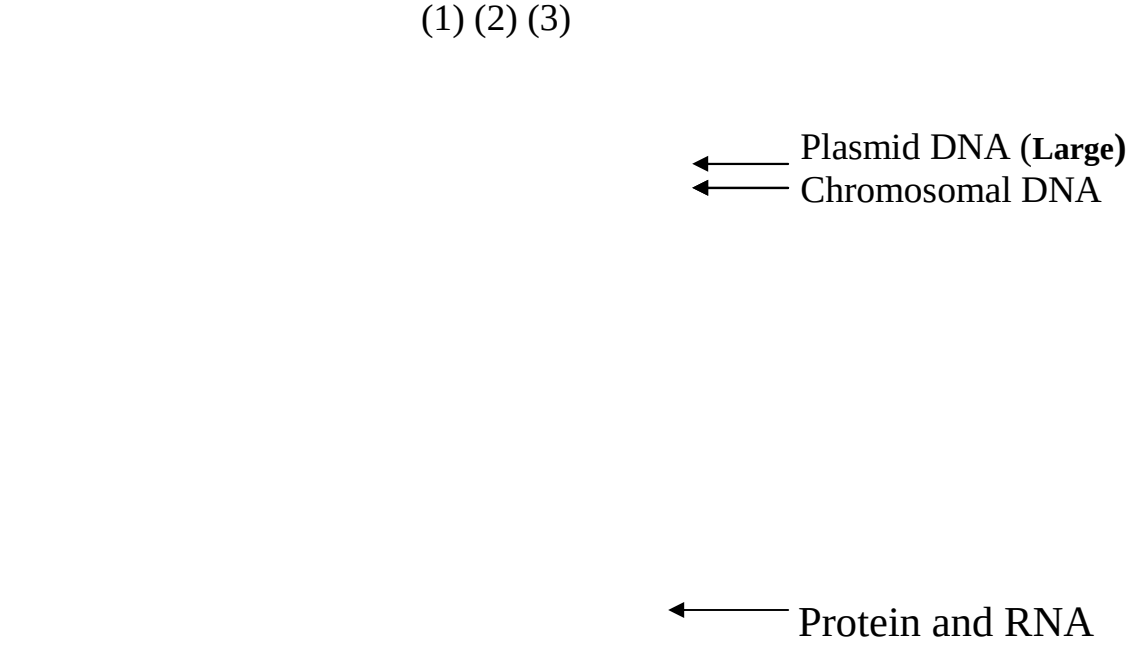

Figure (3-4) Gel electrophoresis of *E. coli* MM 294.

Lane (1and 3) DNA isolation before conjugation
Lane (2) DNA isolation after conjugation

Agarose concentration 0.8 % (w / v).
Voltages 70 V.
Time 6 hours.

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus:

Apparatus	Company (origin)
Autoclave	Gallenkamp (England)
Centrifuge	Gallenkamp
Compound light microscope	Olympus
Cooling centrifuge	Sigma (England)
Distilator	Gallenkamp
Electrophoresis unit	BioRad (Italy)
Haemocytometer	Neubauer (Germany)
Incubator	Gallenkamp
Magnetic stirrer	Gallenkamp
Millipor filters	Sartorins membranes filter GM6H
Oven	Gallenkamp
pH meter	Orient research (U.S.A.)
Refrigerator	Ishtar
Sensitive balance	Mettler (Switzerland)
Shaker incubator	GFL (Germany).
Spectrophotometer	Hitachi (Japan)
U.V. transilluminator	Vilber lourmat (France)
Vortex mixer	Labeco (Germany)
Water bath	Gallenkamp

2.1.2. Chemicals:

Chemical	Company(origin)
$CaCl_2$	BDH
Chloroform	BDH
EDTA	LTD (England)
Ethanol	BDH
Ethidum bromide	Oxoid (England)
Glucose	BDH
Glycerol	BDH
ISopropanol	BDH
KH_2PO_4	BDH
Mg SO_4.7H2O	BDH
Na_2HPO_4	BDH
NH_4Cl	BDH
Peptone	BDH
Phenol	BDH
Potassium acetate	BDH
SDS	BDH
Sodium chloride	Sigma (England)
Sodium hydroxide	Fluka
Tris-HCl	BDH
Urea powder	Oxiod

2.1.3. Bacterial Strain:

Strain	Morphology and genetic characteristics	Source
E. coli MM 294	Rif^r, thi^-, $HsdM^+$, $HsdR^-$	College of science Al-Nahrain University

-thi $^-$: need to thiamin .

-Hsd R : Host specific DNA Restraction.

-Hsd M : Host specific DNA Modification.

-Rif^r: resistance to the Rifampicin.

2.1.4. Enzymes:

Enzyme	Company
Lysozyme	Sigma, USA
Proteinase k	Sigma, USA

2.1.5. Kit:

kit	Company
Api 20E	Central Public Health Laboratory

2.1.6. Antibiotics:

A-Antibiotics disks:

Antibiotics	Symbols	Concentration µg/disc	Company
Ampicillin	Am	10	Oxoid
Chloramphenicol	C	30	
Tobramycin	Tob	10	
Streptomycin	S	10	
Gentamycin	Gm	20	
Clindamycin	CD_2	20	
Amoxicillin	Ax	10	
Carbenicillin	PY	100	
Trimethoprim	Tp	1.25	
Cephalexin	Cfx	30	
Tetracycline	Tc	85	
kanamycin	K	30	
Rifampicin	Rif	100	

B-Antibiotics Powder:

Antibiotic	Company
Ampicillin	Hikma
Amoxicillin sod.	
Nalidixic acid	
Rifampicin	
Gentamycin	

2.1.7. Medium:

Media	Company
Nutrient Agar	Difco
Nutrient Broth	
Urea agar Base	
Simmon citrate Agar	
Tetra- Thionate Broth	
MacConky Agar	Oxoid
Blood agar Base	
Triple sugar Iron Agar	
Brain Heart Infusion Broth	
Salmonella –Shigella Agar (SS agar)	
Brain Heart Infusion Broth	
MR-VP Medium	BDH
Luria – Bertani Broth	Laboratory prepared medium
Luria – Bertani Agar	
Indol Media	
Urease Media	
Blood Agar	
Glucose Fermentation	
Minimal Media M9	

2.1.8. Animals:

Healthy male mice (strain BALB/c) were obtained from the laboratory animal house in biotechnology research center / Al-Nahrain University. Animal ages were ranged between 6 and 8 weeks, their weights were about 20-28g. Animals were placed in small plastic cages, every cage size was 29 × 12.5 × 11.5 cm, and each cage contains 2 mails. Floors of the cages were covered with the soft crushed wood shaving. The cages were washed once a week with soap and tap water and then sterilized with 70% ethyl alcohol through out the period of the study as it was mentioned by Peter and Pearson, (1971) .Animals were kept under suitable environmental conditions such as room temperature which it was maintained between 24-26 ^{O}C exposed to 14 hour day light program daily. The animals were fed with a suitable quantity of water and basal diet (standard pellets) according to Vodopich and Moor, (1992).

2.2. Methods:

2.2.1. Media Preparation:

2.2.1.1. Ready to Use Medium:

All media mentioned were prepared as recommended by the manufacturing company, and sterilized by autoclaving.

- Nutrient Agar
- Nutrient Broth
- MacConky Agar
- Blood agar Base
- Urea agar Base
- Triple sugar Iron Agar
- Simmon citrate Agar
- MR-VP Medium
- Tetra- Thionate Broth
- Brilliant Green sulfar Agar
- *Salmonella –Shigella* Agar (SS agar)
- Brain Heart Infusion Broth

2.2.1.2. Laboratory prepared Media (Sambrook *et al*., 1989):

1-Luria – Bertani Broth:

Component	Concentration(g/l)
Peptone	10
Yest extract	5
NaCl	10

All components were dissolved in 950 ml D.W., pH was adjusted to 7.0, and then the volume was completed to 1000 ml with distilled water, and sterilized by autoclaving.

2-Luria-Bertani agar:

This medium was prepared by adding 15 g of agar to luria-bertani broth before the volume was completed to 1000 ml, then it was sterilized by autoclaving.

3-Indol media:

This medium was prepared by dissolve 1g of peptone in 100ml D.W., and sterilized by autoclaving.

4-Urease media:

This medium was prepared by sterilizing 95 ml of urea-agar base, after cooling to 50 ºC, 5ml of 40 % urea solution (sterilized by filtration) was added then it was poured in test tubes in slant position.

5-Blood agar medium:

This medium was prepared by performing 95 ml of Blood Agar Base and sterilized by autoclaving, after cooling to 50 ˚C, 5 ml of sterilized human blood was added.

6- Glucose fermentation test medium:

This medium was prepared by adding 1 g of glucose to100 ml of brain heart infusion broth, then few drops of phenol red indicator was added, pH was adjusted to 7.2, then the medium was sterilized by autoclaving.

7- Minimal Media M9:

Component	Concentration (g %)
Na_2HPo4	0.6
KH_2PO_4	0.3
NaCl	0.05
NH_4Cl	0.1

All the component were dissolved in 98 ml of D.W., pH was adjusted 7.2 ,and sterilized by autoclaving, after cooling 0.2 ml of 1M $MgSO_4$, 0.01 ml of 1M $CaCl_2$ and 1 ml of 20% glucose (that sterilized by filtration) were added, then complete volume to 100 ml with sterilized distilled water.

8-Sidrophore production medium (Nassif and Sansonetti, 1987)**:**

It was prepared by adding 2 g of agar to 100 ml of M9 medium followed by adding 0.2 M of 2, 2-dipyridyl sterilized by filtration.

7-Medium for motility test (Collee et al., 1996):

This medium was prepared by dissolving 0.1 % agar in nutrient broth. It is important that the final medium should be quite clear and transparent. Dispense 10 ml amounts in test tube and leave to set in the vertical position.

2.2.2. Antibiotics Solutions:

Antibiotic solutions were prepared according to Maniatis *et al*., (1982) and as follows:

1- Ampicillin solution (10 mg/ml):

This solution was prepared by dissolving 1g of ampicillin in 90 ml of distilled water, and then the volume was completed to 100 ml with D.W. and sterilized by filtration.

2- Amoxcillin solution (10 mg/ml):

This solution was prepared by dissolving 1g of amoxcillin in 90ml of D.W., and then volume was completed to 100 ml, and sterilized by filtration.

3- Nalidixic acid solution (2 mg/ml):

This solution was prepared by dissolving 0.2g of the antibiotic in 50 ml of D.W., few drops of absolute alcohol and NaOH (0.1M) was added till the antibiotic was completely dissolved, then volume was completed to 100 ml with D.W., and sterilized by filtration.

4- Rifampicin solution (2.5 mg/ml):

This solution was prepared by dissolving 0.25g of the antibiotic in 90ml of acetone, and then volume was completed to 100 ml with acetone, and sterilized by filtration.

5-Gentamycin solution (40 mg/ml):

This solution was obtained from gentamycine injectable in a concentration 40 mg/ml.

Antibiotics solutions were stored at 4˚C, when they were used they were added to Muller-Hinton agar medium at a final concentration mentioned in table (2-1) according to Maniatis *et al*., (1982); and Hughes *et al*., (1982).

Table (2-1) Antibiotic Stock solution used in this study.

Antibiotics	Symbol	Solvent	Stock conc. (mg/ml)	Final conc.* (µg/ml)
Ampicillin	(Am)	Distilled water	10	100
Amoxicillin	(Ax)	D.W.	10	100
Nalidixic acid	(Nal)	D.W.	2	20
Rifampicin	(Rif)	Acetone	2.5	100
Gentamycin	(Gm)	Injection	40	10

***final concentration of antibiotics was in Brain- Heart agar.

2.2.3. Indicators:

Indicators used in this study were prepared according to (James and Sherman, 1987) and as fallows:

A-Indol (kovac's reagent):

This indicator was prepared by dissolving 5 g of ρ-Dimethyl-aminobenzaldehyde in 75 ml of Isoamyl alcohol**,** and then 25 ml of concentrated HCl was added.

B-Voges-Proskaur (VP):

-VP 1: It was prepared by dissolving 40 g of potassium hydroxide in 100 ml of D.W.

-VP 2: It was prepared by dissolving 5g of α-naphthol in 100 ml of absolute ethanol**.**

2.2.4. Method of Sterilization:

A- Autoclaving:

Culture medium and solutions were sterilized by autoclaving at 121ºC, 15 psi for 15 min.

B- Filtration sterilization:

Solutions that sensitive to heat were sterilized by filtration through Millipore's (0.22 μ m) in diameter.

C- Dry heat sterilization:

Glassware will sterilized by dry heat at 180 ˚C for 3 h.

3.2.5. Samples Collections:

Hundred stool samples were collected from patient child (under 5 years old), suffering from diarrhea, in Al-Kadhimia, Al-Elwia hospitals in Baghdad and Ibn Gaswan hospital in Basra governorates respectively, during the period from Oct, 28, 2005 to Jan, 28, 2006.

Stools samples were taken from different male and female patients using wood sticks, and transferred to test tubes containing 15 ml of sterile peptone water, and then they were incubated at 37˚C for 24h.

2.2.6. Isolation of *Salmonella* (Maniatis *et al*., 1982)**:**

After incubation, 10 ml of cultured stool samples in peptone water were transferred to conical flasks containing sterile tetra-thionate broth (enrichment media for the selection of *salmonella*), and incubated at 37˚C for 24 hours.

Then loopful from each flask was streaking on a selective medium SS agar plates and on brilliant green sulfar agar plates, incubated at 37˚C for 24 h, then the result colonies were suspected to be *Salmonella*, and subjected to further identification.

2.2.7. Identification of *Salmonella typhimuirum:*

2.2.7.1. Morphological Characteristics (Atlas *et al*., 1995):

Colonies that were able to grow on the selective media were further identified by studying their morphological characteristics beginning with staining ability, appearance under light microscope, and size, shape, edge, color and transparency of colony on plates.

2.2.7.2. Biochemical Tests:

The following biochemical tests were achieved for the suspected colonies according to Macfaddin, (1980) and as follows:

1- Motility test (Collee *et al*., 1996)**:**

Test tubes containing semisolid agar media, were inoculated with single colony with a straight wire, making a single stab down the center of the tube to about half depth of the medium incubated at 37 ˚C for 24-28 hrs, motile bacteria was swarmed, it was easily recognized by the naked eye. Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.

2- Indole production test

Test tubes containing 1% peptone broth were inoculated with single colony, and incubated at 37 ºC for 24 -48h , then 0.05 ml of kovac's reagent was added. Presence of red ring on the surface of the medium indicates a positive result.

3- Methyl red test:

Test tubes containing MR-VP broth were inoculated with single colony, and incubated at 37 ºC for 48h. Then 5 drops of methyl red

indicator was added to each tube and mixed, presence of red color indicates a positive result.

4- Voges-Proskaur test:

Test tubes MR-VP broth was inculcated with single colony of each isolate, and incubated at 37 ˚C for 48h. Then 1 ml of VP1 and 3 ml of VP2 was added to 5 ml of culture broth and shacked for 30 seconds. The formation of pink to red color indicates a positive result.

5- Citrate utilization test:

Test tubes containing simmon–citrate agar slant were inoculated by stabbing with single colony of each isolate, and incubated at 37˚C for 18-24 h, changing the color of the medium from green to royal blue indicates a positive result.

6- Ureas test (Atlas *et al*., 1995)**:**

Test tubes containing urease medium were inoculated with single colony of each isolate, and incubated at 37 ºC for 24h, changing the medium color from yellow to pink indicates a positive result.

7- Triple Sugar Iron test (TSI) (Atlas *et al*., 1995)**:**

Test tubes containing triple sugar Iron (TSI) agar slant were inoculated by stabbing with single colonies, and incubated at 37˚C. Results are as following:

Color	Result	
	pH :Slant/Bottom	**Utilization**
Red / Yellow	Alkaline / Acid	Glucose only fermented; Peptones utilization
Yellow / Yellow	Acid / Acid	Glucose fermented; lactose and /or sucrose fermented
Red /Red	Alkaline / Alkaline	No fermentation of glucose, lactose or sucrose / Peptones utilization

While the formation of Blake precipitated indicates H_2S production and pushing agar to the top indicates CO_2 formation.

7- Glucose fermentation:

Inculcated the media that prepared in (2.2.1.2), incubation at 37 ˚C for 18-24h, then examining for the changing in the color from red to yellow as indicator for fermentation.

2.2.7.3. APi 20E Identification for *Salmonella* Isolates:

Identification of the isolates was carried out by sub-culturing representative colonies from MacConkey Agar plates on APi 20E microtubes systems. This system is designed for the performance of 20 standard biochemical tests from a single colony on plate medium. Each test in this system is performed within a sterile plastic microtube which contains the appropriate substrates and is affixed to an impermeable plastic strip (gallery). Each gallery contains 20 microtubes (each of which consists of a tube and a couple selection.

The biochemical tests included in this system are the following:

1-Beta-galactosidase test ONPG.

2- Arginine dihydrolase test ADH.

3- Lysine decarboxylase test LDH.

4- Ornithine decarbxylase test ODC.

5- Citrate utilization test CIT.

6- Hydrogen sulphide test PLS.

7- Urease test URE.

8- Tryptophane deaminase test TDA.

9- Indole test IND.

10- Voges-Proskauer test VP.

11- Gelatin Liquefaction test GEL.

12- Glucose Fermentation test FLU.

13- Manitol Fermentation test MAN.

14- Inositol Fermentation test INO.

15- Sorbitol Fermentation test SOR.

16- Rhamnose Fermentation test RHA.

17- Sucrose Fermentation test SAL.

18- Melibiose Fermentation test MEL.

19-Amygdalin Fermentation test AMY.

20-Arabinose Fen Tientation test ARA.

21-Oxidase test OXI.

-Preparation of the Galleries:

Five ml of tap water dispensed in to the incubation tray to provide a humid atmosphere during incubation.

-preparation of Bacterial Suspension:

Single colony from plating medium was picked, and suspended in 5 ml sterile distilled water by rubbing against the side of the tube and mixed thoroughly with the water.

-Inoculation of the Galleries:

With a sterile Pasteur pipette, the twenty microtubes were inoculated .According to the manufactures instructions both the tube and couple section of CIT, VP and GEH microtubes were filled. After inoculation couple section of the ADH, LDC, ODC, H_2S and URE microtube were completely filled with sterile mineral oil.

-Incubation of the Galleries:

After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18 to 24 hrs at 37ºC.

-Reading of the Galleries

All the reactions not requiring reagents were recorded first, then the following reagents were added to the corresponding microtubes:-

1- One drop of 3.4 % ferric chloride to the TDA micortube .

2- One drop of kavoc's reagent to the IND micortube .

3- One drop of voges-proskauer reagent to VP micortube.

4- One drop of the oxidase reagent to either H_2S or ONPG micortube.

The biochemical reactions performed by the APi 20 E and their interpretations are listed in table (2-2).

Table (2-2) Interpretation of Reactions Performed by APi 20 E.

Microtube	Positive	Negative
ONPG	Yellow	Colorless
ADH	Red/Orange	Yellow
LDC	Orange	Yellow
ODC	Red/Orange	Yellow
CIT	Blue-Green	Pale green/Yellow
H2O	Black deposit	Colorless/Grayish

URE	Red/Orange	Yellow
TDA	Dark brown	Yellow
IND	Red Ring	Yellow Ring
VP	Pink /Red	colorless
GEL	Diffusion of Black pigmen	No diffusion
GLU	Yellow	Blue/Blue green
MAN	Yellow	Blue/Blue green
INO	Yellow	Blue/Blue green
SOR	Yellow	Blue/Blue green
RHA	Yellow	Blue/Blue green
SAC	Yellow	Blue/Blue green
MEL	Yellow	Blue/Blue green
AMY	Yellow	Blue/Blue green
ARA	Yellow	Blue/Blue green
OX	Violet/Dark purple	Colorless/Light purple

-Identification of the isolates:

Identification of the isolate using the analytical profile index (Numerical coding) for rapid identification at species and biotype level were done as supplied by the manufacturer.

For using the index , the biochemical profile obtained have to be transformed into a numerical profile and to compare it with those listed in the index by transform all 21 biochemical results in to a seven – figure numerical profile (seven –digit number) by placing them into groups of three consigning a specific value for each of the positive as follows :

Group 1			**Group2**			**Group3**			**Group 3**		
ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU
1	2	3	4	1	2	3	4	1	2	3	4

Group 7			**Group 8**			**Group 9**		
MAN	LNO	SOR	RHA	SAC	MEL	AMY	ARA	OXI
1	2	4	1	2	4	1	2	4

Each positive reaction is given a value equal to 1, 2 or 4 according to the position of the test in its group. The sum of these three values was given the corresponding figure. Thus, the figure can have a value from 0 to 7 (zero for negative reaction) the seven digits numerical profile is then looked up in the index and the identification is determined.

More Identification assay was done for the local isolates using antisera grouping technique.

2.2.8. Maintenance of Bacterial Isolates:

Maintenance of Bacterial isolates was performed according to Johnson *et al*., (1988) and as fallowing:

A- Short- Term storage (few weeks):

Bacterial isolates were maintained for few weeks on nutrient agar plates; the plates were wrapped tightly with parafilm, and then stored at 4 ºC.

B-Medium-Term storage (1-3months):

Bacterial isolates were maintained for few months by stabbing nutrient agar slants in screw-capped tubes containing 5-8 ml of nutrient agar medium and stored at 4 ºC.

C- Long Term storage:

Single colony was used to inoculated in Braine heart infusion broth and incubated at 37 ºC for 24 hr, and then 8.5 ml of cell suspension was mixed with 1.5 ml of glycerol, and stored at -20 ºC.

2.2.9. Selection of the Most Virulent Isolate:

In order to select the most virulent isolate of *S.typhimurium*, 100 µl of fresh culture of each isolates suspension, optical Density of cell suspension for each isolates was ranged between (0.43-0.47) which equal to 10^8 cell/ml according to macfarlaned tubes that used to infect mice by injection intrapretonially, then the mice were kept under the same conditions of incubation and feed. Animals were then watched and the period for the survived animals after injection was measured till death of each one. Isolate that causes the death in a shortest time was regarded the most virulent isolate.

2.2.10. Antibiotic Sensitivity Test:

A-Standard Disk Diffusion Method:

Susceptibility of the locally isolated *Salmonella typhimurium* to different antibiotics was examined according to standard disk diffusion method described by NCCLS, (1990) and as follows:

1- five ml of sterile brain heart infusion broth was inoculated with 0.1ml of a fresh culture of the local isolated *Salmonella typhimurium* and incubated at 37 ºC for 4 hour with shaking incubator (100 rpm) at 37˚C. Then serial dilutions were prepared, and 0.1 ml of the fourth dilution 10^{-4} was spread on brain heart infusion agar plates in 3different planes by rotating the plate approximately 60º each time to obtain an even distribution of the inocula.

2- The inoculated plates were placed at room temperature for 30 min to allow absorption of excess moisture, and then the selected antibiotic disks were placed on the inoculated plates (5 disks/plate) and incubated at 37ºC for 18 hour in an inverted position.

3- After incubation the diameter of inhibition zones were measured and compared with standards according to the National Committee for Clinical Laboratory Standards (NCCLs, 1990).

B- Pour Plate Method (Prescott *et al*., 1999)**:**

1- On hundred ml of brain heart infusion agar was prepared and autoclaved.

2- After cooling to 50 ˚C, an appropriate concentration of each antibiotic was added at a final concentration that described in table (3-1), then mixed gently and poured in sterile plate.

3- *Salmonella typhimurium* were streaked on the above plates.

4- The inculcated plates were incubated at 37 ˚C for 24 hrs, and then examined for the growth of bacteria.

2.2.11. Haemolysin Production (De Boy *et al*., 1980):

The ability of the locally isolates *Salmonella typhimurium* toproduce haemolysin was examined on blood agar plates prepared in (2.2.1.2). This test was performed by streaking single colony on blood agar plates, and incubated at 37 ºC for 24 hour. Appearance of clear zone or haemolysis around the colonies indicates a positive result.

2.2.12. Sidrophore Production (Johnson *et al*., 1988)**:**

Ability of locally isolated *Salmonella typhimurium* for sidrophore production was detected on sidrophore production medium prepared in

(2.2.1.2). First of all sidrophore agar plates were inoculated with single colonies of the bacterial isolate, and then they were incubated at 37 ˚C for 24 hours and checked for growth. *S. aureus* was used as a negative control.

2.2.13. Assay of Liver Enzyme GOT & GPT:

To detect the effect of the local isolates of *Salmonella typhimurium* on the activity of mice liver enzymes (GOT and GPT), five animals for each treatment were infected by injection 100µl of *S. typhimurium* intrapretonially. Fifteen mice were used for this experiment and were divided to subgroups, each group containing five animals and as follows:

1- Group 1: Mice in this group were injected with 100 µl of PBS and regarded as a positive control.
2- Group 2: Mice in this group were injected with 100 µl of *Salmonella typhimurium* containing 10^5 cell/ml.
3- Group 3: Mice in this group were injected with 100 µl of cured *Salmonella typhimurium* containing 10^5 cell/ml.

Before death of the treated mice because of infection with the virulent isolates, the blood was taken by heart punicher and the serum was separated from the blood and used. Activity of liver GOT and GPT were assayed at the same time fore each group and as the fallowing:

-Procedure:

According to Reitman and Frankel, (1957), blood samples were collected from the mice by heart puncture .The serum was separated by centrifuging at 5000 rpm for 10 min., and then the serum was taken and treated as follows:

Two test tubes were used for each sample, the first one contained the

blank reagent and second one contains the sample. These samples were treated as in the following:-

	GPT	**GOT**
Reagent 1 Reagent 2	1 ml —	— 1 ml
Incubate for 5 min at 37°C.		
Serum	0.2 ml	0.2 ml
Mix and incubate at 37°C	— 1 hour	— 30 min.
Reagent 3	1 ml	1 ml
Mix. Let stand for 20min at room temp		
0.4 N NaOH	10 ml	10 ml
Mixed and wait four 5 min. Measure at wave length 510 nm.		

2.2.14. Plasmid Profile:

DNA isolation was performed using modified alkaline lysis methods of Birnboin and Doly described by Lavery *et al*., (1997) and as follows:

A- Solutions:

1- Tris- EDTA-Glucose solution (TEG)

This solution was prepared to be consisting of:

25 mM Tris –HCl

10 mM EDTA

50 mM Glucose

PH was adjusted to pH 8 and sterilized by autoclaving for 10 min and stored at 4 ºC.

2- Alkaline sodium dodecyl sulphate (0.2 N NaOH -1% SDS):

This solution was prepared by dissolving 1g of SDS in 100 ml of freshly prepared 0.2 N NaOH solutions.

3- Potassium acetate solution (Maniatis *et al*., 1982)

This solution was consisted of the followings:

60 ml of 5M potassium acetate

1.5 ml of glacial acetic acid

28.5 ml of Distilled water

PH of this solution was adjusted to pH 4.8.

4- Tris-EDTA buffer solution (TE-buffer)

20 ml of this solution was prepared to be consisting of:

50 mM Tris –HCl

1 mM EDTA

PH of this solution was adjusted to pH=8.

5-Phenol solution (Sambrook *et al*., 1989):

Crystalline phenol was melted in a water bath at 68 ºC, then the soluble phenol was extracted several time with equal volumes of phenol buffer (0.01 M Tris base and 0.01 M Na_2 EDTA), until the pH of the aqueous phase was 8.0.

6-Phenol –chloroform solution:

This solution was prepared by mixing 2 volumes of phenol with 1volume of chloroform, and stored in a closed dark bottle at 4 ºC.

7-Loading buffer:

4 mg of bromophenol blue was dissolved in 20 ml of glycerol, then the volume was completed to 100ml with D.W. .

8-Ethidium Bromide solution (10 mg /ml):

This solution was prepared according to Maniatis *et al*., (1982) by dissolving 0.1 of ethidium bromide in 10 ml of distilled water**.**

9- Tris Borate-EDTA (TBE) buffer solution (10 X) (Maniatis *et al*., 1982).

250 ml of this solution was prepared to be consisting of:

27 g of Tris – base

89 mM boric acide

2.3 mM Na_2-EDTA

The working solution is 1X.

B- Enzymes:

1- lysozyme solution (20 mg / ml):

This solution was prepared by dissolving 20 mg of lysozyme dissolved in 1ml of TEG solution as prepared in (2.2.14).

2- proteinase K solution (5 mg / ml):

This solution was prepared by dissolving 5 mg of proteinase K dissolved in 1 ml of TEG solution as Prepared in (2.3.14).

C-Procedure:

1- Falcon tubes containing 5 ml of Brain heart infusion broth was inoculated with a single colony of the locally isolate *Salmonella typhimurium*, and incubated over night at 37 ºC.

2- Transferred 1.5 ml of overnight culture to ependorff tubes, then it was centrifuged at 6000 rpm for 5 min at 5ºC.

3- The pellet was resuspented in 0.2 ml of TEG solution.

4- 100 µl of lysozyme solution was added, and incubated for 1 h at 37 ºC.

5- 100 µl of proteinase K solution was add, and incubated for 30 min.

6- Cells were completely lyses by the addition of freshly prepared 0.2 N NaOH -1% SDS.

7- Mixed by gentle inversion and left to stand in ice bath for 5 min.

8- 0.3 ml of 5M potassium acetate was added, mixed by inversion, and left to stand in ice bath for a further 15min.

9- Lyses cell was centrifuged at 14000 rpm for 5min at 4 ºC, and then the supernatant was transferred to new micro centrifuge tube.

10- DNA was extracted with phenol –chloroform solution by adding equal volume of the supernatant then centrifugatin at 14000 rpm at 5 ºC for 5min was done.

11- The supernatant was transferred to a new microcentrifuge tubes, and the DNA was precipitated by adding two volumes of cold absolute ethanol, then left to stand for 1h. Then centrifuged at 14000 rpm at 5 ºC for 5min.

12- The DNA precipitate was washed with 1 ml of 70% ethanol, and then centrifuged at 14000 rpm at 5 ºC for 5min.

13-After centrifugation, the supernatant was discarded, and the precipitated DNA was dried and dissolved in 20 µl of TE buffer solution.

2.2.15. Agarose Gel Electrophoresis (Sambrook *et al*., 1989):

Agarose gel was prepared by dissolving 0.7 gm of agarose in 100 ml of TBE buffer 1X, and heated on hot plate till all agarose crystals were dissolved. After cooling to 50 ºC it was poured gently in the apparatus tray and cooled to 25 ºC. Then the tray containing agarose gel was transferred and immersed in apparatus tank containing TBE buffers solution.

Samples of DNA were mixed with 1/1 volume of the loading buffer and added to the wells in gel. Generally, gels were run for 6 hrs at 5 volt/cm and the gel buffer added up to the level of horizontal gel surface. Agarose gel was then stained by immersing in 0.5 μg/ml ethidium bromide for 30-45 minutes.

DNA bands were visualized by UV-illumination at 302 nm on an UV-transilluminator. Gels were distained in distilled water for 30-60 minutes to gel ride of background before photographs were taken.

2.2.16. Curing of Plasmid DNA:

Curing experiment was performed by using Ethnidium Bromid according to Trevors, (1986) and Salzano *et al*., (1992) and as follows:

A- Solutions:

10 mg/ml of Ethidium Bromide stock solution was prepared by dissolving 0.2g of Ethidium Bromide in (20) ml of D.W. stock solution will sterilized by filtration .

B-Procedure:

1- Single colony of the locally isolated *Salmonella typhimurium* was used to inoculate 5 ml of luria broth and incubated at 37 ºC for 3.5 hours. Then 0.1 ml a liquates was taken from the growth culture and used to inoculate universal tubes containing 5 ml of brain-heart infusion broth and specific concentration of ethidium bromide solution in each tube (50, 100, 200, 300, 400, 600, 800, 1000, 1200, 1400, 1600 μg/ml). All the tubes were incubated with shaking (150 rpm) at 37 ºC for 24 hours.

2- The growth density of different universals was measured visually and compared with the control to determine effect of each curing agent on bacterial growth.

3- The lowest concentration of each curing agent that inhibited bacterial growth was considered as the minimum inhibitory concentration (MIC).

4- Samples were taken from universals containing the highest concentration of each curing agent that still allows bacterial growth, which is known as (sub lethal concentration).

5- Serial dilutions from the tubes containing growth and sub-lethal concentration of ethidium bromide was achieved, then 0.1 ml of 10^{-8} dilution was spread on Braine-heart infusion agar plates, and incubated at 37 ºC for 24 hours.

2.2.17. Selective of Cured Cell:

After treatment with curing agent, 100 colony that still able to grow on braine-heart infusion agar were selected randomly and were replica plated (using tooth pick) on brain-hear infusion agar plates containing the antibiotic to which the wild isolate was resist. Plates then incubated at 37 ºC for 24 hours, the resultant colonies may by arise from cured cells (Trevors, 1986).

2.2.18. Conjugation:

Conjugation was achieved according to the method described by Weiservar *et al*., (1987) and modified by Nasir, (2000) and as following:

1- Conical flasks contain 50 ml of Luria broth were inoculated with 0.1 ml of *E. coli* MM 294 and 0.1 ml of *Salmonella typhimurium* , then incubated at 37 ºC for 18h.

2- Tubes contain 5ml of Luria broth were inoculated with 0.2ml of the Donor and 0.2 ml of the recipient, and incubation at 37 ºC for 4h.

3- Mixed cultures of both isolates and then incubated at 37 ºC for 3h, after that, cells were centrifuged at 6000 rpm for 15 min.

4- Wash the pellet of cells with 10 mM $Mg\ So_4$ then resuspend with the same solution.

5- Filtered the cells with filter paper (0.95μm), then put the paper at the surface of dish containing MacConky agar and incubation at 37 ºC for 18h.

6- Removed the filter paper and washed the growth with 2 ml of Luria broth by mixing until have homogenized solution.

7- Then serial dilution was done and cultured 0.1ml from dilution on plate containing selective media that permit growth of conjugative cell and not grow the recipient and donor cell. Then incubation at 37 ºC for 24-48h.

2.2.19. Statistical Analysis:

A one way analysis of variance was performed to test whether group variance was significant or not, the comparison between groups were used analysis of variance test (ANOVA) (Almohammed *et al*., 1986).

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اكملت البكلوريوس عام ٢٠٠٤ بمعدل ٦٨٠٨

اكملت السنه التحضيريه للماجستير بمعدل ٧٦٠٧

1. Introduction & Literature review

1.1. Introduction:

Salmonella is a member of the family *Enterobacteriaceae*, gram negative, rod shape, consist of more than 2200 serovars, and infect a wide variety of humans and animals through the world, there is a widespread occurrence in animals, particularly in poultry and swine, certain environmental sources of the organism include water, soil, insects, kitchen surfaces, animal feces, raw meats, raw poultry, and raw seafood (Sallyers and Whitt, 1994).

Salmonellosis is the name of gastrointestinal tract disease caused by infection with *Salmonella typhimurium*, it is more common in the summer than winter. Children are the most likely to get salmonellosis (Bean *et al*., 1990).

Salmonella typhimurium is abroad-host pathogen, a major cause of food poisoning disease, although the infection usually results in a self-limiting gastroenteritis, more systemic infections do occur and can result in fever and other complications, even death (Helmuth *et al*., 1985).

Multiple resistant strain of *Salmonella typhimurium* come from plasmids containing drug resistance genes are well known in bacteria and can be transferred among different species of enteric bacteria. However, genes coding for resistance to these antibiotics in *Salmonella typhimurium* are located on chromosomal and plasmid DNA. Further more, a relatively high frequency of hyper mutable strains among pathogenic *Salmonella* spp. could be found (Le Clerc, 1996).

Salmonella typhimurium is a complicated pathogen that has created, evolved or acquired numerous virulence factors that contribute to its

overall pathogenesis, analysis of the molecular mechanisms of *Salmonella typhimurium* found that virulence factors responsible for pathogenecity are often encoded by plasmids (Slauch, 1999).

Most *S. typhimurium* isolates carry a plasmid about 90 kb. It will refer to this plasmid simply as the virulence plasmid of a particular host strain, the most apparent consequence of virulence plasmid carriage is to enhance the growth rate of the bacterium during the systemic phase of disease; this phenotype is conferred by an 8-kb region of the plasmid that encodes the *spv* (*Salmonella* plasmid virulence) genes (Helmuth *et al*., 1985; and Gulig *et al*., 1993).

Aims of the study:

1- Isolation and identification of *Salmonella typhimurium* from children stool samples.
2- Select the most virulent isolates according to its ability to infect and kill mice animals.
3- Studying antibiotic resistance, sidrophore and hemolysin production by the selected isolate.
4- Studying plasmid profile of the selected isolate and determine the role of plasmid in bacterial pathogencity.

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Abbreviation	Meaning
SDS	Sodium Dodecyl Sulphat
EDTA	Ethelene–Dramine Tertra acetrc acid
LB	Luria – Bertani
U.V.	Ultraviolet
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvate transaminase
NCCLS	National Committee for Clinical Laboratory Standards

References

(A)

-Aabo, S.; Rasmussen, O. F.; Rossen, L.; Sorensen, P. D.; and Olsen, J. E. (1993). *Salmonella* identification by the polymerase chain reaction. Mol. Cell. Probes. 7:171-178.

-Al-Hayali, C. M. (1993). *Salmonella* in Iraq, A five year review 1985-1989. Diploma thesis. Saddam College of Medicine. Saddam University. Iraq.

-AL-Mohammed, N. T.; AL-Rawi, K. M.; Younis, M. A.; and AL-Morani, W. K. (1986). Principles of Statistics. AL-Mosil University. Iraq.

-AL-Obaidy, L. A. (2001). Investigating the antimutagenic effect in two extracts from *Urtica pilufera* plant in white mouse. M. Sc. Thesis. College of Science. University of Kufa. Iraq.

-Al-Shallchi, S. A. (1999). The plasmid profile of some *E. coli* isolates that cause urinary tract infections. Biotechnol. 1: 36-45.

-Aly, S. M. (2003). Pathology of Salmonellosis among ostrich chicks in Egypt. Suez Canal University. Ismailia. Egypt.

-Al-Zaag, A. (1987). Cloning and molecular characterization of hemolysin and β-glycosidase of *Citrobacter freundii*. Ph.D. Thesis. University of Queezland. Iraq.

-Al-Zaag, A. (1994). Molecular biology for bacterial virulencity. Al-Qabes printing. Bagdad. Iraq. (In Arabic)

-Amieva, M. R. (2005). Important bacterial gastrointestinal pathogens in children: a pathogenesis perspective. Pediatr. Clin. North Am. 52(3): 749-77.

-Amyes, A. S; Hall, S. S.; and Smith, H. (1989). The transmissible nature of genetic factor in *E.coli* that control haemolysin production. J. Gen. Microbiol. 47: 153-161.

-Anderson, E. S.; and Lewis, M. J. (1965). Drug resistance and its transfer in *Salmonella typhimurium*. Nature. 206: 579-583.

-Atlas, R. N.; Brown A. E.; and Paks, L. G. (1995). Laboratory Manual Experimental Microbiology. 1st edition. Mosby. USA.

(B)

-Babu, J. P.; Abraham, S. N.; and Dabbous, M. K. (1986). Interaction of a 60-kilodalton D-mannose-containing salivary glycoprotein with type1 fimbriae of *Escherichia coli*. Infect. Immun. 54: 104-108.

-Banatvala, N.; Cramp, A.; Jones, I. R.; and Feldman, R. A. (1999). Salmonellosis in North Thames (East), UK: associated risk factors. Epidemiol. Infect. 122 (2): 201-207.

-Bean, N. H.; Griffin, P. M.; Goulding, J. S.; and Ivey, C. B. (1990). Foodborne disease outbreaks, 5-year summary, 1983-1987. Mortal Wkly Rep. CDC Surveill Summ. Mar. 39(1): 15-57.

-Benenson, A. (1996). Salmonellosis. In: Control of Communicable Diseases Manual. American Public Health Association. 410-414.

-Bergmeyer, H. U. (1974). Methods of Enzymology Analysis. Vol I. Academic Press. New York. USA.

-Berry, L. J.; and Smythe, D. S. (1964). Effects of bacterial endotoxins on metabolism. VII. Enzyme induction and cortisone protection. J. Exp. Med. 120: 721-732.

-Presscott, L. M.; Harley, J. P. and Klein, D. A. (1999). Microbiology. 4th edition, McGraw-Hill.

-Brenner, F. W.; Villar, R. G.; Angulo, F. J.; Tauxe, R.; and Swaminathan, B. (2005). *Salmonella* nomenclature. J. Clin. Microbiol. 38: 2465-2467.

-Bhatia, R.; and Inchhpujam, R. L. (1994). Immunization against infections diseases. First Edition. Medial Publishers. LTD.

-Birnboim, H.; and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7:513-523.

-Bonnefoi, M.; Hasim, M.; Sauvagnac, P.; Burgat, V.; and Braun, J. (1989). Liver enzymes changes in a Guinea pig model of facial eczema (sporidesmiotoxicosis). Enzyme. 42: 39-46.

-Bouanchaud, D. H.; Scavizzi, M. R.; and Chabbert, Y. A. (1969). Elimination by ethidium bromide of antibiotic resistance in Enterobacterceae and Staphylococci. J. of General Microbio. 54: 417-425.

-Boyd, E. F.; and Hartl, D. L. (1997). Recent horizontal transmission of plasmids between natural populations of *Escherichia coli* and *Salmonella enterica*. J. Bacteriol. 179: 1622-1627.

-Boyd, E. F.; and Hartl, D. L. (1998). *Salmonella* virulence plasmid. Modular acquisition of the *spv* virulence region by an F-plasmid in *Salmonella enterica* subspecies I and insertion into the chromosome of subspecies II, IIIa, IV and VII isolates. Genetics. 149: 1183-1190.

-Brown, L.; and Elliott, T. (1997). Mutations that increase expression of the *ropS* gene and decrease its dependence on *hfg* function in *Salmonella typhimurium*. J. Bacteriol. 179(3): 656-662.

-Brown, N. F.; Coombes, B. K.; Brumell, J. H.; and Finlay, B. (2003). Structure-function analysis of sifa, an effecter traslocated by the SPI2-Encoded type III Secretion system. University of British Columbia. Vancouver. Canada.

-Brumella, J. H.; Steale–Mortimer, O.; and Finlay, B. B. (1999). Bacterial invasion: force feeding by *Salmonella*. Curr-Biol. 9 (8): 277-280.

-Bullen, J. J.; Rogers, H. J.; and Griffiths, E. (1987). Role of iron in bacterial infection. Cur. Top. Microbiol. Immunol. 80: 1-35.

(C)

-Carramin~nana, J. J.; Young uela J.; Blanco, D.; Rota, C.; Agustin, A.; and Herrera, A. (1997). Potential virulence determinations of *Salmonella* serovrs from poultry and human sources in Spain. Vet. Microbiol. 54 (3): 375-383.

-Chen, Y. H.; Chen, T. P.; Tsia , J. J.; Hwang , K. P.; Lu, P. L.; Cheng, H. H.; and Peng, C. F. (1999). Epidemiological study of human Salmonellosis during 1991-1996 in southern Taiwan. Kao-Hsiung-I-Husuch-KO-Tsa –Chih. 15(3): 127-136.

-Clewell, D.; Yagi, Y.; Dunny, G.; and Schult, A. (1974). Characterization of three plasmid DNA molecules in a strain of *Streptococcus faecalis*. Identification of a plasmid determining erythromycin resistance. J. Bacterial. 117: 283-289.

-Collee, J. G.; Barrie P.; Andrew, G.; Auttony, M.; and Tsimmons, F. (1996). Test for identification of bacteria. Mackie and McCarthy. Livingstone, New York.

-Coynanlt, C.; Robbe-Saule, V.; Popoff, M. Y.; and Norel, F. (1996). Growth phase and *spvR* regulating of transcription of *Salmonella typhimurium spv ABC* virulence gene. Microbpatho. 13: 133-143.

-Cruickshank, R.; Duguid, J. P.; Marmion, B. P. and Swain, R. H. A. (1975). Medical Microbiology. The practice of Medical Microbiology. 12th. Churchill Livingstone. London.

(D)

-D'Aoust, J. Y. (1989). "*Salmonella*" pp.327-445 In Doyle, M.P. (ed). "Food-Borne Bacterial pathogens". Marcel Dekker. Inc. New York and Basel.

-De Boy, J.; Wachsumth, K.; and Davis, B. (1980). Haemolysin activity in enterotoxigenic and non-enterotoxigenic strain of *E.coli*. J. Clin. Microbiol. 12: 193-198.

-D'Mello, D.; Mehto, D.; Pereira, J.; and Rao, C. (1999). A toxicity study of simultaneous administration of tamoxifen and Diazepam to female Wister rats. Exp. Toxicol. Pathol. 51: 549-553.

(E)

-Eggman, S.; Lofdah, S.; and Burmangl. (1997). An allelic variant of the chromosomal gene for class A beta-lactamase K2, specific for *Salmonella* is ancestor of SHV-1. Antimicrob. Agents chemother. 41: 2705-2709.

(F)

-Falchi, G.; Canu1, N. A.; Uzzau1, S.; Chitsatzo, O.; Gwanzura, L.; Pietro, C.; and Rubino, S. (2003). Multidruge resistance in *Salmonella* spp. University of Sassari. Sassari. Italy.

-Fang, F.C.; Libby, S. J.; Buchmeier, N. A.; Loswen, P.C.; and Switala, J. (1992). The alternative sigma factor (*ropS*) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA. 89: 11978-11982.

-Fenwick, R. G.; and Curtiss, R. (1973). Conjugal deoxyribonucleic acid replication by *E. coli* k-12: effect of chloramphenicol and rifampicin. J. Bacteriol. 116: 1224-32.

-Fierer, J.; Echmann, L.; Fang, F.; Peifer, C.; Finaly, B. B. and Guiney, D. (1993). Expression of the *Salmonella* virulence plasmid gene *spvB* in cultured macrophages and non phagocytic cells. Infect. Immun. 61: 5231-5236.

-Finaly, B. and Falkow, S. (1988). Virulence factors associated with *Salmonella* species. Microbiological Sciences. 5 (11): 324-327.

-Finlay, B.; Heffron, F.; and Falkow, S. (1989). Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. Science. 243: 940-943.

(G)

-Gadeberg, O. and Biom, J. (1986). Morphological study of the *in vitro* cytotoxic effect of alpha-hemolytic *E. coli* bacteria and culture supernatant on human blood granulocytes and monocytes. Actapathol. Immun. Scan. 94: 75-83.

-Galan, J. E. (1996). Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. Apr: 20(2): 263-71.

-Gale, T. (2005). Conjugation from World of Microbiology and Immunology. Microbiology and Immunology. (Review)

-Gazouli, M.; Tzelepi, E.; Markogiannakis, A.; Legakis, N. J.; and Tzouvelekis, L. S (1998). Two novel plasmid –mediated cefotaxime –hydrolyzing beta –lactamase (CTX-M-5 and CTX-M-6) from *S. typhimurium*. Microbiol. Lett. 165: (2): 289-393.

-Geddes, J. K. (2003). A functional screen to identify type III secreted proteins in *Salmonella typhimurium*. Oregon Health and Sciences University. Portland.

-Giannella, R. A.; Formal, S. B.; and Dammin, G. J. (1973). Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. J. Clin. Invest. 52:441.

-Griffiths, E.; Chart, H.; and Stevenson, P. (1988). High-affinity iron up take systems and bacterial virulence, p. 121-137. In. J. A. Roth (ed.), virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.

-Groves, D. L. (1979). A temperature gradient technique for the elimination of antibiotic resistance. Canada. J. Microbiol. 24: 1476-84.

-Guiney, D. (1997). Regulation of bacterial virulence gene expression by the host environment. J. Clin. Invest. 99 (4): 565-569.

-Guiney, D. G.; Fang, F. C.; Krause, M.; Libby, S.; Buchmeier, N. A. and Fierer, J. (1995). Biology and clinical significance of virulence plasmids in *Salmonella* serovars. Clin. Infect. Dis. 5: 146-51.

-Gulig, P. A. and Curtiss III, R. (1988). Cloning and transposon insertion mutagenesis of virulence genes of the 100-kilobase plasmid of *Salmonella typhimurium*. Infect. Immun. 56: 3262-3271.

-Gulig, P. A.; and T. J. Doyle. (1993). The *Salmonella typhimurium* virulence plasmid increases the growth rate of *Salmonella* in mice. Infect. Immun. 61: 504-511.

-Gulig, P. A.; Danbara, D. G.; Guiney, A. J.; Norel, F.; and Rhen, M. (1993). Molecular analysis of *s*pv virulence genes of *Salmonella* virulence plasmids. Mol. Microbiol. 7: 825-830.

-Gulig, P.; and Curtiss III, R. (1987). Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. 55: 2891-2898.

-Gulig, P.; Caldwell, A.; and Chiodo, V. (1992). Identification, genetic analysis and DNA sequence of a 7.8 kb virulence region of the

Salmonella typhimurium virulence plasmid. Mol. Microbiol. 6 (10): 343-348.

-Guo, L.; Lim, K. B.; Gunn, J. S.; Bainbridge, B.; Darveau. R. P.; Hackett, M.; and Miller, S. I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP/phoQ*. Science. 276 (53): 250-253.

(H)

-Hassan, M. A. K. (2002). The use of some plant extracts for inhibition of genotoxic effects for some anticancer drugs in mouse .Ph. D. Thesis. College of Science. University of Babylon. Iraq.

-Heffernan, E. J.; Harwood, J.; Fierer, J.; and Guiney, D. (1992). The *Salmonella typhimurium* virulence plasmid complement resistance gene *rck* is including *pagC* and *ail*. J. Bacteriol. 174: 84-91.

-Helmuth, R.; Stephen, R.; Bunge, C., Hoog, B.; Stienbeck, A.; and Buling, E. (1985). Epidemiology of virulence –associated plasmid and outer membrane protein patterns with in seven common *Salmonella* serotypes. Infect. Immun. 48(1): 175-182.

-Hensel, M.; Nikolaus, T.; and Egelseer, C. (1999). Molecular and functional analysis indicates a mosaic structure of *Salmonella* pathogenicity island 2. Mol. Microbiol .31(2): 489-498.

-Heurtin, C.; Donnio, P. Y.; Perrin, M.; Travert, M. F.; and Avril, J. L. (1999). Increasing incidence and comparison of nalidixic acid resistance *Salmonella entrica* sub spp. *entricia* serotype *typhimurium* isolates from humans and animals. J. Clin. Microbiol. 37(1): 266-269.

-Hohn, B.; and Korn, D. (1969). Segregation of a sex factor with the *E. coli* chromosome during curing by acridine orange. J. Mol. Biol. 45: 385-396.

-Holt, J. G; krieg, N. R.; Sneath, H. A; Staley, J. T.; and Williams, S. T. (1994). Bergey's manual of determinative bacteriology. 9th ed. Williams and Wilkins. USA.

-Hughes, C.; Phillips, R.; and Roberts, A. (1982). Serum resistance among *E. coli* strains causing urinary tract infection in relation to O type and the carriage of haemolysin, colicin and antibiotic resistance determinants. Infect. Immun. 35:270-275.

(I)

-Ikeda, J. S.; and Hirsh, D. C. (1985). Common plasmid encoding resistance to ampicillin, chloramphenicol, gentamycin and trimethoprin–sulfadiazine in two serotypes of *Salmonella* isolated during an out break of equine Salmonellosis. Am. J. Vet. Res. 46 (4): 769-773.

(J)

-Jacoby, G. A., and Sutton, L. (1991). Properties of plasmids responsible for production of plasmids responsible for production of extended-spectrum B-Lactamase. Antimicrob. Agents Cheother. 35:164-169.

-James, G. and Sherman, N. (1987). Microbiology: A laboratory Manual. The Benjamin / Cummings Publishing Co. California. USA.

-Jawetz, E.; Melnick, J. L.; and Adelberg, E. A. (1998). Ch.15.In: Review of medical microbiology.13th ed. Lange Medical Publication. California.

-Johnson, J. R. (1991). Virulence factor in *Escherichia coli* urinary tract infection. Clin. Microbiol. Res. 4 (1): 80-128.

-Johnson, J. R.; Moseley, S. L.; Roberts, P. L.; and Stamm, W. E. (1988). Aerobactin and other virulence factor genes, among strains of

Escherichia coli causing urosepsis: association with patient characteristics. Infect .Immun. 56: 405-412.

-Jones, B. D.; and Falkow, S. (1996). Salmonellosis: host immune responses and bacterial virulence determinants. Annu. Rev. Immunol. 14:533-561.

-Jones, G. W.; Rebert, D. K.; Svinarich, D. M.; and Whitfield, H. J. (1982). Association of adhesive, Invasive, and virulent phenotypes of *Salmonella typhimurium* with Autonomous 60-Megadalton plasmids. Infect. Immun. 38 (2): 476-486.

(K)

-Khimji, P. L.; and Miles, A. A. (1978). Microbial iron-chelators and there action on *Salmonella* infection in guinea-pigs. Br. J. Exp. Pathol. 59: 133-143.

-Koupal, L. R.; and Deibel, R. H. (1975). Assay characterization and localization of an enterotoxin produced by *Salmonella* Infect. Immun. 11: 14-22.

-Krause, M.; Roudier, C.; Fierer, J.; Harwood, J.; and Guieny, D. (1991). Molecular analysis of the virulence locus of the *Salmonella dublin* plasmid pSDL2. Mol. Microbiol. 5: 307-316.

(L)

-Larvery, A.; Rossney, A. S.; Morrison, D.; Power, A. and Keane, L. T. (1997). Incidence and detection of multidrug resistant *enterococci* in Dublin hospitals. J. Med. Microbiol. 46: 150-156.

-Le Clerc. J. E.; Li, B.; Payne, W. L.; and Cebula, T. A. (1996). High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science. 274: 1208-1211.

-Livrelli, V.; Champs, C.; and Mrtino, P. (1996). Adhesive properties and antibiotic resistance of *Salmonella*, *Klebsilla*, *Enterobacter* and *Serratia* clinical isolates involved in nosocomil infection. J. Clin. Microbiol. 34 (4): 1963-1969.

-Low, J. C.; Hopkins, G.; King, T.; and Munro, D. (1996). Antibiotic resistant *Salmonella typhimurium* DT104 in cattle. Vet. Rec. 138: 650-651.

(M)

-Macfaddin, J. F., (1980). Biochemical tests for identification of medical bacteria 2nd Ed. Williams and Wilkins 428E. Preston Street. Baltimore. Md 21201, USA.

-Mahon, B. E.; Slutsker, L.; Hutwagner, L.; Drenzek, C.; Maloney, K.; Tommey, K.; and Griffin, P. M. (1999) . Consequences in Georgia of a nation wide out break of *Salmonella* infections: what you don't know might hurt you. Am. J. Public. Health. 89 (1):31-35.

-Malkawi, H. I. and Youssef, M.T. (1996). Characterization of *Escherichia coli* isolated from patients with gastrointestinal infections in northern Jordan: Antibiotic resistance and plasmid profiles. J. Res. 11: 172-192.

-Maniatis, T.; Fritch, E.F.; and Sambrook, J. (1982). Molecular cloning a laboratory manual. Cold spring Harbor Laboratory, cold spring harbor, New York.

-Marcus, S. L.; Brumell, J. H; Pteifc, C. G.; and Finlay, B. B. (2000). *Salmonella* pathogencity island big virulence in small packages. Microbes and infection. 2: 1-12.

-Mathur, V.; and Dive, P. (1981). Effect of MPG on the radiation induced changes in the intestinal activity of ALP in mice. Natl. Acad. Scie. Lett. 4: 183-185.

-Matloob, M. H. M. (2000). Genetic study on *Salmonella typhimurium*. Ph. D. theses. College of Science. Bagdad University. Iraq.

-Mayeux, P. R. (1997). Pathobiology of lipopolysaccaride. J. Toxical. Environ. Health. 51(5): 415-435.

-Molnar, J. (1988). Antiplasmid activity of tricyclic compounds. Meth. And Fin. Exp. Clin. Pharmacol. 10(7): 467-474.

-Molnar, J.; Beladi, I.; and Holland, I. B. (1978). The plasmid curing action of imiparmine in *E. coli* K12. Genet. Res. 31: 197-201.

(N)

-Nakamura, M.; Sato, S.; Ohya, T.; Suzuki, S.; and Ikeda, S. (1985). Possible relationship of a 36-megadalton *Salmonella enteriditis* to virulence in mice. Infect. Immun. 47: 831-833.

-Nasir, R. B. (2000). The role of *P. aruginosa* in utilizing of hydrocarbon compounds. M.Sc. Thesis. College of Science. Al-Nahrain University. Bagdad University. Iraq.

-Nasir, R. B.; Al-Gelawi, M. H.; and Ali, N. A. (2004). The role of *P. aruginosa* RB19 plasmids in utilizing of hydrocarbon compounds. J. of Al-Nahrain University (Science). Vol: 7 No 1.

-Nassif, X. and Sansonetti, P. (1987). Bacterial iron uptake synthesis: their role in virulence. Bull. Inst. Pasteur. 85: 307-327.

-National Committee for Clinical Laboratory Standards (NCCLS). (1990). Performance standards for antimicrobial disk susceptibility tests. 4th ed. Approved standard M2-A_4. National Committee for clinical Laboratory Standards. Villanova, Pa.

-Norel, F.; Pisano, M.; Nicoli, J.; and Popoff, M. (1989) .A plasmid – borne virulence region (2-8kb) from *Salmonella typhimurium* contains two open reading frames. Res. Microbiol. 140: 627-630.

-Norel, F.; Robbe-Saule, V.; Popoff, M. Y. and Coynault, F. C. (1992). The putative sigma factor kat F (RopS) is required for the transcription of *Salmonella typhimurium* virulence gene *spvB* in *E. coli*. FEMS Microbiol. 78: 271-276.

(O)

-Ofek, I.; and Dolye, R. J. (1994). Bacterial adhesion to cells and tissues. Champman and Hall. Ltd. London. United Kingdom.

-Old, D. C.; and Adegbola, R. A. (1985). Antigenic relationships among type-3 fimbriae of *Enterobacteriaceae* revealed by immunoelectron microscopy. J. Med. Microbiol. 20: 113-121.

-Ottow, J. C. G. (1975). Ecology, physiology, and genetics of fimbriae and pilli. Annu. Rev. Microbiol. 29: 79-108.

-Ou, J. T.; Baron, L. S.; Dai, X. Y.; and Life. C. A. (1993). The virulence plasmids of *Salmonella* serovars typhimurium, choleraesuis, dublin, and enteritidis, and the cryptic plasmids of *Salmonella* serovars copenhagen and sendai belong to the same incompatibility group, but not those of *Salmonella* serovars durban, gallinarum, give, infantis, and pullorum. Microbiol. Pathog. 8: 101-107.

(P)

-Peter, W.; and Pearson, A. E. G. (1971). The laboratory animals principles and practice. Academic press. New York. Pp: 226-234.

-Poppe, C.; Smart, N.; Khakhria, R.; Johnson, W.; Spika, J.; and Prescott, J. (1998). *Salmonella typhimurinm* DT104: a virulent and drug-resistance pathogen. Canada. Vet. J. 39(9): 559-565.

-Pratt, D. S.; and Kaplan, M. M. (2001). Evaluation of liver function. In: Harrison's Principle of International Medicine. McGraw-Hill Company. New York. USA.

-Pyen, S. (1988). Iron and virulence in the family *Enterobacteriacea*. CRC Cit. Rev. Microbiol. 16: 81-111.

(R)

-Rasool, S. A.; Ahemed, A; and Khan, S. (2003). Plasmid born antibiotic resistance factors among indigenous *Salmonella*. Pak. J. Bot. 35(5): 243-248.

-Reeves, M. W.; Evins, G. M.; Heiba, A. A.; Plikaytis, B. D.; and Farmer, J. I. (1989). Clonal nature of *Salmonella typhi* and genetic relatedness to other *Salmonella* as shown by proposal of *Salmonella bongori* comb. Nov. J. Clin. Microbiol. 27: 313-320.

-Reitman, S.; and Frankel, A. S. (1957). A colorimetric method for the determination of serum glutamic oxaloacetatic and glutamic pyruvic transaminases. Amer. J. Clin. Path. 13: 234-240

-Rhen, M.; O'Conner, C. D.; and Sukupolvi, S. (1992). The outer membrane permeability mutation of the virulence–associated in *tra* T–like gene. FEMS Microbiol. Lett. 552:145-154.

-Ricci, G.; and Federici, G. (1982). Method for determination of transaminases and relative diagnostic kit, United States patent.

-Rice, L. B.; Carias, L. L.; and Bonomo, R. A. (1996). Molecular genetic of resistance to both ceftazidime and beta-Lactamase inhibitor combinations in *K. pneumonia* and *in vivo* response to betalactam therapy. J. Infect. Dis. 173: 151-158.

-Ringertz, S.; Bellete, B.; Ohman, G.; and Krovall, G. (1990). Antibiotic sensitivity of *E.coli* isolated from in patients with urinary tract infections in hospitals in Addis papa and Stockholm. Bulletin as a world health organization. 68: 61-68.

-Rioux, C. R.; Friedrich, M. J.; and Kadner, R. J. (1990). Genes on the 90Kilobase plasmid of *Salmonella typhimurium*. Confer low–affinity cobalmin transport: Relation ship to fimbria biosynthesis genes. J. Bacteriol. 172:6217-6222.

-Roudier, C.; Fierer, J.; and Guiney, D. G. (1992). Characterization of translation termination mutation in the *spv* operon of the *Salmonella* virulence plasmid pSDL2. J. Bacteriol. 174: 6418-6423.

-Ruize, J.; Castor, D.; Goni, P.; Santamaria, J. A.; Borrego, J. J.; and Vila, T. (1997). Analysis of mechanism of quinobone resistance nalidixic acid resistant clinical isolates of *Salmonella typhimurium*. J. Med. Microbiol. 46: 623-628.

(S)

-Sallyers, A. A.; and Whitt, D. D. (1994). Bacterial pathogenesis. A molecular approach. Washington. DC: 255-260.

-Salzono, G.; Villani, F.; Pepe, O.; Sorrentino, E.; Moschetti, G.; and Coppola, S. (1992). Conjugal transfer of plasmid-borne bacteriocin production in *Enterococcus faecalis* 226NWC. FEMS Microbiol. 99: 1-6.

-Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular a Laboratory manual. Cold Spring Harbor Laboratory, cold Spring Harbor. New York .

-Sandefure, P. D.; and Peterson, J. W. (1976). Isolation of skin permeability factors from cultures filtrates of *Salmonella typhimurium*. Infect. Immun. 14: 671-679.

-Sanderson, K. E. (1996). F-mediated conjugation, F^+ strains, and Hfr strains of *Salmonella typhimurium* and *Salmonella abony*, p. 2406-2412. *In* Neidhardt, F. C.; Curtiss III, R.; Ingraham, J. L.; Lin, K. B.; Low, E. C. C.; Magasanik, B.; Reznikoff, W. S.; Riley, M.; Schaechter, M.; and Umbarger H. E. (ed.), *Escherichia coli* and *Salmonella*. Cellular and Molecular biology. 2^{nd} ed. ASM Press. Washington, D.C.

-Sanderson, K. E.; and Roth, J. R. (1988). Linkage map of *Salmonella typhimurium*. Edition VII. Microbiological Rev. 52(4): 485-532.

-Sanderson, K. E.; Kadam, S. K.; and MacLachlan, P. R. (1983). Depression of F factor function in *Salmonella typhimurium*. Can. J. Microbiol. 29:1205-1212.

-Shamman, N.; Kadri, K.; Rahmat, A.; and Nagah, W. (1999). Vitamin-C and aloevera supplementation protect from chemical hepatocarcinogenesis in the rats. Nutrition. 14: 846-852.

-Shea, J. E.; Hensel, M.; Gleeson, C.; and Holden, D. W. (1996). Identification of virulence locus evading a second type III secretion system in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA, 93 (6): 2593-2597.

-Slauch, J. (1999). Mechanisms of pathogenesis of *Salmonella*: linking in vitro, animal and human studies. Department of Microbiology. University of Illinois. Urbana.

-Snyder, I. S.; Deters, S. M.; and Ingle, J. (1971). Effect of endotoxin on pyruvate kinase activity in mouse liver. Infec. Immun. 4: 138-142.

-Stein, M.; Leung, K.; Zwich, M.; Portillo, F.; and Finlay, B. (1996). Identification of *Salmonella* virulence gene required for formation glycoprotein with in epithelial cells. Mol. Microbiol. 20(1): 151-164.

-Stephen, J.; Wallis, T. S.; and Starkey, W. G. (1985). Salmonellosis: in retrospect and prospect. p.175. In Evered D, Whelan J (eds). Microbial. Toxins and Diarrhea Disease. Ciba Foundation Symposium 112. Pitman Press. London.

-Stone, G. G.; Oberst, R. P.; Hays, M. P.; Mcvey, S. and Chengappa, M. M. (1994). Detection of *Salmonella* serovars from clinical samples by environmental both cultivation-PCR procedure. J. Clin. Microbiol. 32(7): 1742-1749.

-Sukupolvi, S.; Vuorio, R.; Qi, S. Y.; and Rhen, M. (1990). Characterization of the *tra*T gene and mutants that increase outer membrane permeability from the *Salmonella typhimurium* virulence plasmid. Mol. Microbiol. 4: 47-57.

-Swetwiwathana, A.; Chungsamanukool, P.; Phunbualuang, M.; and Bangtrakulnonth, A. (1994). Studies on the microbiological quality of meet products sold in Bangkok. King Monkut's Agricultural J. 12: 15-24.

(T)

-Taira, S. and Rhen, M. (1989). Identification and genetic analysis of *mkaA* - a gene of the *Salmonella typhimurium* virulence plasmid necessary for intracellular growth. Microbiol. 5: 307-316.

-Taira, S.; Konen, P.; Saarilaht, S.; Sukupolvi, S.; and Rhen, M. (1991). The *mkaC* virulence gene of the *Salmonella* serovar *typhimurium* 96

Kb plasmid encodes a transcriptional activator. Mol. Gen. Genet. 228: 381-384.

-Terakado, N.; Sekizaki, T.; Hashimoto, K. and Naitoh, S. (1983). Correlation between the presence of a fifty–megadaltion in *Salmonella dublin* and virulence in mice. Infect. Immun. 41:443-444.

-Threlfull, E. J.; Frost, J. A.; Ward, L. R.; and Rowe, B. (1996). Increasing spectrum of resistance in multiresistant *Salmonella typhimurium*. Lancet. 347: 1053-1054.

-Tietz, N. W.; Wekstein, D. R. and Shey, D. F. (1986). Text Book of Clinical Chemistry. 2nd ed. W.B. Sunders Company. London.

-Tomoeda, M.; Inuzuka, M.; and Kubo, N. (1968). Effective elimination of drug resistance and sex factors in *E. coli* by SDS. J. Bacteriol. 95: 1078-89.

-Trevors, J. T. (1986). Plasmid curing in bacteria. FEMS Microbiol. Rev. 32: 149-157.

(V)

-Venegas, M. F.; Navas, E. L.; and Gaffny, R. A. (1995). Binding of type1-piliated *Escherichia coli* to vaginal mucus. Infect. Immun. 63: 416-422.

-Vodopich, D.; and Moor, K. (1992). Biology Laboratory manual. 3rd Edition. 112-124.

(W)

-Wallis, T. S.; and Galyv, E. E. (2000). Molecular basis of *Salmonella*-induced enteritis. Mol. Microbiol. 36(5): 997-1005.

-Weiservar, M.; Hubacek, J.; Brenner, V.; Piruzian, E. S.; Kobec, N. S.; and Velikodvorshaya, G. A. (1987). Transposition of Bacterial Gene in the transmissible plasmid. Folia Microbiol. 32: 368-375.

-White, D. G.; Zhao, S.; Simjee, S.; Wagner, D. D.; and McDermott**,** P. F. (2002). Antimicrobial resistance of foodborne pathogens. Microbes Infect. 4: 405-412.

-Wilson, J. A.; Dolye, T. J.; and Gulig, P. A. (1997). Exponential phase expression of *spv*A of *Salmonella typhimurium* virulence plasmid: induction in intracellular salts medium and intracellularly in mice and cultured mammalian cells. Microbiology. 143: 3827-3839.

-Wong, C. K.; Ooi, V. E.; and Aug, P. O. (2000). Protective effect of seaweeds against liver injury caused by carbon tetrachloride in rats. Chemosphere. 41: 173-176.

-Woolcock, J. B. (1984). Pathogenesis of bacterial infections: some determinants of virulence in gram negative bacteria. Aust. Vet. J. 62 (6): 177-181.

(Y)

-Young, H. K.; and Ltillyear, J. K. (1994). Trimethoprim resistance in urinary pathogens in northern Scotland: epidemic spread of a resistance plasmid encoding the type to trimethoprim-resistance dihydrofocte reductase. J. Med. Microbiol. 41(5): 343-348.

Web Sites

-Internet 1:

http://gsbs.utmb.edu/microbook/ch021.htm.

-Internet 2:

http://www.britannica.com/bcom/eb/article/3/0,5716,66793+1+65109,00.

html?query=salmonella%20spp.

-Internet 3:

http://gsbs.utmb.edu/microbook/ch021.htm.

-Internet 4:

http://en.wikipedia.org/wiki/Bacterial_conjugation.

Summary

In this study, one hundred stool samples were collected from children under age of five years of both sexes suffering from diarrhea infection cases from Al-Kadhimia and Al-Elwia hospitals in Bagdad governorate, and from Ibn-Ghaswan hospital in Basra governorate. Stool samples were cultured on *Salmonella-Shigella* agar plates, and then 38 bacterial isolates were obtained. All these isolates were subjected to morphological, microscopically examinations and biochemical tests. Results showed that 10 isolates were *Salmonella tytphimurium*, which further identified using Api-20E and antisera test.

Each of these isolates injected intrapretonally in mice to select the most virulent isolate, that capable to kill the animals in the shortest period. Results showed that *Salmonella typhimurium* SM9 was the most virulent isolates capable to kill the animals within 19 hours after injection in mice.

Ability of SM9 resist antibiotics was examined, and it was found that this isolate was able to resist ampicillin, amoxicillin, gentamycin, streptomycin, tetracycline, cephalexin, nalidixic acid, and kanamycin; while it was sensitive to rifampicin, clindamycin, carbencillin, tobramycin, and trimethoprim. Ability of SM9 to produce hemolysin and sidrophore was also examined, and it was found that this isolate was sidrophore producer; while it was unable to produce hemolysin.

Plasmid profile of SM9 was studied by extraction the total DNA by alkaline lysis method, then electrophoresis on agarose gel. Results showed that this isolate harboring a large plasmid (mega plasmid) that may be confer to some virulence factors. Curing of plasmid DNA was achieved using ethidium bromide to know the role of this plasmid in the

virulence of this isolate, and its ability to antibiotic resistance. It was found that this plasmid was carrying genes conferring resistance to ampicillin, amoxicillin, gentamycin, tetracycline, and kanamycin, in addition to the genes coding for virulence that responsible for the pathogenecity, because of inability of the cured cells to kill mice that injected, rather than its inability to affect the mice liver functions. Liver ability to produce GPT and GOT did not affected blood stream in comparison with control animals. While the wild type (virulent isolate) cause liver dysfunction in lever of mice that led to increase the levels of both enzymes in blood stream of mice, the activity of GPT was increased from 199.3 U/L (in control animals) to 375.1 U/L in injected mice with wild type cells SM9, also the activity of GOT was increased from 68.0 U/L (in control animals) to 90.8 U/L in mice injected with the wild type cells of SM9.

In order to know the ability of conjugative plasmid of SM9 to transfer to member of other *Enterobactereaceae*, conjugation on solid medium by modified filter mating procedure was achieved between SM9 (donor) and the standard strain *E. coli* MM294 (recipient), and after culturing on selective medium, number of transforming cells were received the antibiotic resistant traits to ampicillin, amoxicillin, gentamycin, tetracycline, and kanamycin. Plasmid profile of transconjugants was examined, and it was found that there was a plasmid band after electrophoresis on agarose gel, in addition to chromosomal band in comparison with the wild type of *E. coli* MM 294, that refer to virulence plasmid of SM9 isolate was a conjugative plasmid.

الاهـداء

الى منقذِ البشريةِ من الجهلِ وباني مدينةِ العلمِ الرسول الاعظم صلى الله عليه واله و صحبة وسلم **.....**

الى وطنٍ تجذرت فيه الحضاراتُ منذُ آلاف السنين فأنبتتْ علوماً ومعارفَ اثمرت ازدهاراً للبشريةِ جمعاء .....

الى رفقةِ دربٍ لن انسى لهم ما قدموه لي ما حييتْ .....

الى هؤلاء اقدمُ جهدي الذي يشكلُ مستقبلَ حياتي

بسم الله الرحمن الرحيم

فَبدأ بِأوعيتهِم قَبلَ وِعاءِ أَخِيهِ ثمَ استَخرجَهَا مِن وِعَاءِ أَخِيهِ كَذَلكَ كِدنَا لِيُوسُفَ مَا كَانَ لِيَأخُذَ أَخاهُ فِي دِينِ المَلكِ إلّا أَن يَشَاءَ اللّهُ نَرفعُ دَرَجَاتٍ مَّن نَّشَاءَ وَفَوقَ كُلِ ذِي عِلمٍ عَليمٌ

صدق الله ألعلي العظيم

سورة يوسف

الآية ٧٦

الخلاصة

تم في هذة الدراسة جمع ١٠٠ عينة خروج من اطفال مصابين بالاسهال تحت سن الخمس سنوات و من كلا الجنسين فى كل من مستشفيات الكاظمية و العلوية في محافظة بغداد و مستشفى ابن غزوان في محافظة البصرة. و بعد زرع العينات في وسط سالمونيلا-شكيلا الصلب، تم الحصول على ٣٨ عزلة بكتيرية، وقد اخضعت جميع العزلات للفحوصات المظهرية و الكيموحيوية. وقد شخصت ١٠ من هذة العزلات على انها *Salmonella typhimurium* باستخدام العدة التشخيصية Api 20E .

حقنت جميع العزلات في الحيوانات المختبرية كلا على انفراد لانتقاء العزلة الاكثر ضراوة التى تؤدي الى قتل الحيوان باقصر مدة ممكنة. وقد اشارت النتائج الى ان العزلة البكتيرية *Salmonella typhimurium* SM9 كانت هي الاكثر ضراوة، اذ ادت الى قتل الحيوان بعد مرور ١٩ ساعة من الحقن في الفئران المختبرية. اختبرت قابلية العزلة SM9 على مقاومة المضادات الحياة، وقد اشارت النتائج الى ان هذة العزلة كانت مقاومة للامبسلين، اموكسيلين، جنتامايسين، حامض النالدكسيك، ستريبتومايسين، تتراسايكلين، سيفالكسين، و الكانامايسين؛ في حين كانت حساسة للرافامبسين، كلندامايسين، كاربنسيلين، توبرومايسين، و التراي مثبريم. وقد اشارت النتائج الى ان هذة العزلة SM9 كانت تخلق لسايدروفور في حين كانت غير منتجة للهيمولايسين.

درس النسق البلازميدي العزلة SM9 باستخلاص الدنا الكلي بطريقة التحلل القاعدي ثم الترحيل على هلام الاكاروز وقد اظهرت النتائج ان هذة العزلة تحتوي على بلازميد كبير (ميكا بلازميد) ربما يشفر لبعض عوامل الضراوة. و لدى اجراء عملية تحييد الدنا البلازميدي باستخدام مادة بروميد الايثيديوم لمعرفة دور البلازميدات في ضراوة هذة العزلة، ومقاومتها لمضادات الحياة، وجد بان هذا البلازميد يحمل الجينات المسؤولة عن صفة المقاومة لكل من امبسلين، اموكسيلين، جنتامايسين، تتراسايكلين، كانامايسين، كما انة يحمل جينات الضراوة المسؤولة عن امراضية البكتريا، وذلك لعدم قدرة الخلايا المحيدة بعد حقنها في الفئران المختبرية على قتل مضايفها من ناحية ، من ناحية اخرى عدم قدرتها في التاثير على وضائف الكبد، كما لم تتاثر كفاءة الكبد في انتاج انزيمات GPT و GOT الى مجرى الدم مقارنة بحيوانات السيطرة، في حين تمكنت خلايا النوع البري (العزلة الضارية) من تدمير وظائف الكبد والذي ادى الى ارتفاع مستويات كلا الانزيمين في دم الفئران المختبرية،اذ ازدادت فعالية انزيم GPT من 199.3 وحدة\لتر (في حيوانات السيطرة) الى 375.1 وحدة\لتر في الحيوانات التي حقنت بخلايا النوع البري للعزلة SM9 .

و لمعرفة القابلية الاقترانية لبلازميد هذة البكتريا على الانتقال الى البكتريا المعوية الاخرى فقد اجريت عملية الاقتران البكتيري على الاوساط الغذائية الصلبة بطريقة التزاوج الغشائي المحورة بين العزلة المحلية SM9 (الواهبة) و السلالة البكتيرية القياسية *E. coli* MM 294 (المستلمة) و بعد التنمية على الاوساط الغذائية الانتقائية تم الحصول على عدد من المقترنات البكتيرية التي اكتسبت صفة المقاومة لمضادات تتراسايكلين، للامبسلين، اموكسيلين، جنتامايسين، و كانامايسين. وعند التحري عن النسق البلازميدي للمقترنات البكتيرية لوحظ وجود حزمة دنا بلازميدي بعد الترحيل الكهربائي على هلام الاكاروز، فضلا عن وجود حزمة دنا كروموسومي، مقارنة بالنوع البري لبكتريا *E. coli* MM 294 مما يشير الى ان بلازميد العزلة المحلية SM9 هو من النوع الاقتراني.

Republic of Iraq
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Role of Conjugative Plasmid in The Virulence of locally Isolated *Salmonella typhimurium* .

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By

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May 2007

Chapter One

Introduction & Literature Review

Chapter two

Materials & Methods

Chapter Three

Results & Discussion

Chapter Four

Conclusions & Recommendations

References

وزارة التعليم العالي والبحث العلمي
جامعة النهرين/ كلية العلوم
قسم التقانة الاحيائية

دور البلازميدات الاقترانية في ضراوة بكتريا *Salmonella typhimurium* المعزولة محليا.

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

سحر مدحت حسين

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٤

جمادي الاول ١٤٢٧هـ

نيسان ٢٠٠٧ م