# I N T R O D U C T I O N

The existance of plasmid in Salmonella strains was just a speculation untile 1970; soon afterwards, several workers identified a large plasmid in strain of Salmonella typhimurium,however, with few exception no phenotype could be ascribed to the plasmid. Hence, the plasmid was called "cryptic plasmid" (Sheehy et al., 1973) .

Recent studies have indicated that large molecular mass plasmids are required for the virulence of several serotypes of Salmonellae which are capable of producing systemic diseases in man and animals. These include S.typhimurium, S. dublin, S. enteritidis, S. choleraesuis and S. paratyphi C. (Barrow and Lovell, 1986 ).

The detailed characterization of these plasmids and the exact role in pathogenesis is still a matter of debate (Paul, 1990) .

2

# AIM OF THE WORK

The present work aimed at

1. Confirmation of the presence of plasmid in Salmonella typhimurium strain .
2. Examination of the contribuation of the plasmid (if present) in Salmonella typhimurium virulence .

# P L A S M I D S

Plasmids are defined as extrachromosomal genetic elements capable of autonomous replication. They exist as convalently closed double-stranded circular deoxyribonucleic acid (DNA), ranging from 0.1% to 5% of the size of the bacterial chromosome (Calos and Miller, 1980).

Plasmids are widely distributed in nature, and most bacteria isolated from clinical materials will contain at least one, and often as many as six, different plasmids. They accur in both Gram-positve and Gram-negative bacteria and may be categorized according to the functions they encode (Gottesman, 1981).

Bacteria, like other living organisms, are continually exposed to a range of noxious agents, such as ultraviolet-irradiation, mutagenic elements, heavy metals, toxic organic compounds, antibiotics and lytic viruses (bacteriophages). Resistance to such agents is, however, widespread and is frequently plasmid determindable (Walker, 1984).

Plasmids also encode for production of pili, which are involved in the adherence of bacteria to epithelial cells. Haemolysin production by E. coli, enterotoxins responsible for gastroenteritis, and many exotoxins such as those responsible for botulism, tetanus and scarlet fever, are also encoded in plasmids (Foster, 1980). Other plasmids code for the ability to metabolize new sugars and carbon

sources, thereby blurring the biochemical distinctions between different species. Some additional properties encoded in plasmids include nitrogen-fixing enzymes and the ability to metabolize hydrocarbons (Calos and Miller, 1980).

In general, plasmids are not necessary for cellular maintenance, but they often provide useful metabolic capabilities (Foster, 1983) .

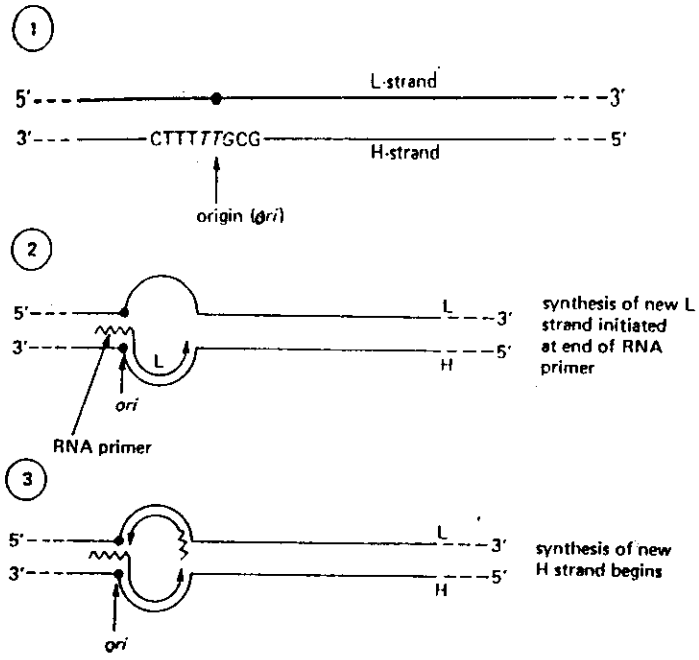
Molecular Weight :

Plasmids molecular weights range from 1 x $10^6$ to about 200 x $10^6$ daltons,Kleinschmidt developed a very useful method for examining DNA molecules by electron microscopy (Bedbrook et al., 1979) . Plasmid molecular weights can also be determined by sedimentation through sucrose gradients or by agarose gel electrophoresis. In both methods, the various forms of plasmid DNA migrate at different rates, so it is important to ensure that the correct form of plasmid is compared with an appropriate standard which is included in sucrose gradient or agarose gel. To determine the positions of plasmids after agarose gel electrophoresis, the gels are stained with ethidium bromide. This binds to the DNA and fluoresces when illuminated by ultraviolet light (Norrander et al., 1983).

Replication :

Plasmid replication begins with synthesis of an ribonucleic-acid (RNA) primer by host RNA polymerase. As the primer transcript is elongated through the ori region (origin

**Figure (1) :** Plasmid replication




(Kimber, 1981).

of replication), the ori DNA becomes unpaired and most recently synthesized part of the transcript with one of the strands. RNase H nicks the primer, and host DNA polymerase I extends the processed primer to begin synthesis of the first strand. Initation of DNA synthesis is negatively regulated by another-encoded RNA (called RNAI) which hybridize the primer RNA. Pairing of the primer RNA to RNAI is thought to change the secondary structure of the primer RNA is some way that makes it unable to pair with ori region DNA. Formation of RNA I-primer RNA hybrid is somehow facilitated by the action of Rop protein (encoded by plasmid's rope gene). So plasmids do not contain an intact rop gene, are thus maintained in higher copy number than plasmids with intact rop gene (Ausubel et al., 1987) (Figure 1) .

The mechanism which ensure that each daughter cell receives a copy of a newly replicated plasmid at cell division are not understood, but it is thought that plasmids become attached to sites on the cytoplasmic membrane which segregate into daughter cells (Bedbrook et al., 1979).

Classification :

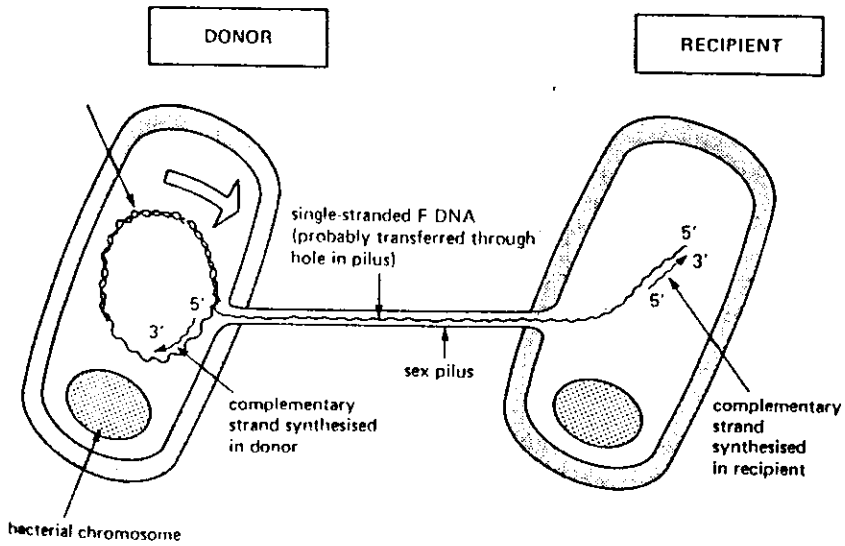
Plasmids may be conveniently classified into two major classes : 1- Conjugative and 2- Non- conjugative (Obbink, 1979) . Conjugative plasmid is self transmissible from one cell to another. Its presence in bacterial cell is usually manifested by synthesis of a proteinaceous cellular

appendage called sex pilus as well as other plasmids mediated proteins which together provide for cell to cell transmission (Novick, 1969) . Non-conjugative plasmids do not have the inherent ability to initiate self transfer nor they encode for sex pilus or other transfer specific proteins (Meynell, 1973).

The first property of a plasmid was discovered by Tantum and Lederberg in 1946, They recognised the ability of the F (fertility) plasmid to transfer chromosomal genes between strains of Escherichia coli. It was subsequently found that the F plasmid could also transfer itself to strains of E. coli (Novick, 1969) .

Donor cells contain an F plasmid (a self-transmissible plasmid ) possesses 15 to 25 genes (called the tra-for transfer- genes) encoding various structures and enzymes for conjugation to occur (Gottesman, 1981). One essential structure encoded by one of the tra genes is the sex pilus, a filament composed of a single polymerized polypeptide (called pilin) extending out from the cell surface, which can be up to 20 um long. Although the sex pilus is essential for transferring DNA by conjugation, it is not clear whether it acts as a long thin tube through which DNA pass from doner to recipient, or whether its primary function is to contract to bring the two cell walls close together (Calos and Miller, 1980). During conjugation, a single - stranded molecule of FDNA is transferred into the recipient, in which the complementary

Figure (2) : Transfer of plasmid DNA during conjugation




(Willetts and Skurray, 1980) .

strand is synthesized (Figure 2) (Willetts and Skurray, 1980).

Ausubel et al. (1987), classified plasmid replicators based on their copy number into :

1. **High-Copy-Number Plasmids (non-Self-Transmissible Plasmids):**

These tend to be under relaxed control. These plasmids sometimes called relaxed plasmids, are relatively small (3 to 20 million daltons) and are present in high numbers (10 to 60 copies) within the bacterial cell. These plasmids have a replicon function, meaning that a specific segment of their DNA serves as an origin for DNA replication. Thus, their replication does not use the same enzymes and is not subject to the same controls as those that function for chromosome replication and the initation of their replication occurs much more frequently than chromosome replication. Moreover, their replication does not require a period of protein synthesis allowing considerable amplification of these plasmids following inhibition of protein synthesis. High-copy number plasmids usually do not have any mechanism to ensure correct segragation of the plasmid to daughter cells, they do not carry the tra genes, but many can be transmitted by conjugation if they are present in a cell that also contains a self-transmissible plasmid.

## 2. Low-Copy-Number Plasmids (Self-Transmissible Plasmids)

These are usually under stringent control. They are large (40 to 100 million daltons), and are replicated by the same enzymes and with the same controls as chromosomal replication. They are present in one to three copies per cell and replicate in synchrony with chromosome. Such plasmids carry the tra genes encoding for sex pili and the DNA transfer enzymes. Most plasmids under stringent control contain sites on their DNA called par (for partition) loci which in some way enable the plasmid copies to be correctly segragated to daughter cells with very high efficiency.

Bacteria often contain two or more different plasmids which are stably inherited, they are said to be compatible with each other. However, certain pairs of plasmids are incompatible, if both are added to a suitable host bacterium, by conjugation or some other means, they are unable to co-exist. After only a few generation most the daughter cells contain only one of the two types of plasmid (Kenneth et al., 1986) .

Incompatibility is used to classify plasmids into groups, any two members of the same group cannot stably co-exist in the absence of selection pressure. Plasmids found in entero bacteria have been classified into about 25 incompatibility groups. There are seven incompatibility groups of staphylococcus plasmids and at least eleven groups of pseudomonas plasmids. As many as seven compatible plasmids can be maintained in E. Coli in the absence of

selection pressure (Chang and Cohen, 1978) .

The molecular basis of incompatibility is unknown, but it appears to be a consequence of the mechanisms controlling plasmid replication and segregation at cell division, where first, plasmid DNA replication of these plasmids is negatively controlled by RNA I which acts in trans on other plasmids with the same primer RNA and second, that these plasmids lack a mechanism to ensure that each plasmid in a cell replicates once per cell cycle (Bolivar et al., 1977).

## Cloning Vectors :

During the 1970s, many plasmids were constructed in the laboratory with fragments of DNA from these naturally accuring plasmids. These artifical plasmids and their derivatives are the most commonly used vectors in recombinant DNA work. All plasmids used as a cloning vectors contain three common feactures : a replicator, a selectable marker and a cloning site.

The replicator is a strech of DNA that contain the site at which DNA replication begin (usually called the origin of replciation or ori and that also includes genes encoding RNAs and proteins which are necessary for replication

The selectable marker is usually dominant and is usually a gene encoding resistance to some antibiotics.

The cloning site is a restriction endonuclease cleavage site, into which foreign DNA can be inserted without interfering with the plasmid's ability to replicate or to confer the selectable phenotype onits host (Norrander et al., 1983) .

Foreign DNA from any source can be treated with the same restriction enzyme, cleaving it into fragments of various sizes, each possessing sticky ends as the cleaved plasmid DNA. The foreign DNA fragments are then mixed with the cleaved plasmid DNA and the entire mixture is annealed so that the sticky ends will pair at random. (Stoker et al., 1982) . Treatment with poly nucleotide Ligase will convalently link the paired fragments yielding, closed circle of plasmid DNA into which a fragment of foreign DNA has been inserted. (Norrander et al., 1983). Such hybrid plasmids can be introduced by transformation into bacterial host, where th foreign fragment of DNA will be replicated and transcribed along with the plasmid DNA ( Ausubel et al. 1987) .

For example, the plasmid PBR 322 encodes for genes that confer resistance to ampicillin and tetracylcine . Cleavage of this plasmid with the restriction enzyme Hind III (derived from Haemophilus influenza) accurs within the gene conferring resistance to tetracylcine. Thus, when this plasmid and foreign DNA are cleaved with Hind III and the resulting hybrid plasmids used to transform E. coli, only cells receiving the plasmid will be resistant to ampcillin.

Such resistant clones can then be screened for tetracycline sensitivity. In this case, only cells containing a plasmid with an insert of foreign DNA will be sensitive, because closure of the plasmid without an insert would regenerate the original plasmid, conferring resistance to tetracycline (Hitchkiss, 1974) .

**R Plasmids** :

R plasmids were discovered in Japan in 1957. Until about 1950, bacillary dysentry in Japan was treated with sulfonamide, although this drug was rapidly becoming less effective because an increasing proportion of Shigella strains were sulphonamide resistant

Tetracycline, streptomycin and chloramphenicol were also used to treat dysentry from about 1950, but by 1957 a small proportion (about 2%) of Shigella were found to be resistant to one or more of these antibiotics. The proportion of resistant strains increased to 13% by 1960, the multiply resistant strains became the predominant type of Shigella in Japan

The first indication that the multiple resistance might be plasmid-specified came from analysis of strains of Shigella responsible for epidemics. Both multiply resistant and fully sensitive forms of the same strain were sometimes isolated during an epidemic. Indeed, both forms were

sometimes isolated from individual patients. Multiply resistant strains of E. coli were sometimes isolated from patients excreting resistant Shigella, so Akiba and Ochiai suggested that resistant E. coli might transfer DNA coding for drug-resistance to Shigella (Davies and Smith, 1978).

Following the Japanese discovery of R plasmids in enterobacteria, they were soon found in strains isolated from many parts of the world. They accur in both Gram positive and Gram negative bacteria and have been found in almost all species which are pathogenic for man and animals. R plasmids are particularly common in the enterobacteria, such as E. coli , salmonella and shigella and in Staphylococci. (Calos and Miller, 1980).

Two important features of R plasmids which have constributed to their rapid evolution and dispersal are their conjugative ability and the presence within their sequences of genetic elements known as transposons (Silvtr, 1978) .

The term transposon is the general name for a variety of DNA fragments (coding for drug resistance, toxins, or nothing) that can move from one site to other target sites in the same or a different DNA molecule by a process called transposition. It range in size from about 2000 to 2500 base pairs (Calos and Miller, 1980) .

Transposons are incapable autonomous replication, hence, they are replicated only when integrated into a plasmid, phage, or bacterial chromosome (Kenneth et al., 1986).

R-plasmids mediated resistance is generally due to synthesis of protein which may enzymatically destroy the drug, modify the antibiotic to an innocuous form or interact with the cell envelope to make it impermeable to the antibiotics (Davies and Kagan, 1977).

R-Factors may determine resistance to one antibiotic or they may carry resistance to 10 or more distinct antimicrobial agents (Kenneth et al., 1986) .

Penicillin-resistant strains of Staphylococcus aureus were rapidly selected after pencillin came into use in the 1940s. By 1946, 14% of strains in hospitals (where penicillin was extensively used) were penicillin resistant. This proportion rose to 38% in 1947 and to 59% in 1949. Almost all S. aureus strains isolated from hospital now are penicillin-resistant, they produce an inducible B-lactamase. In retrospect, it seems that R plasmids were responsible for much of the penicillin -resistance which became apparent in the late 1940s. Plasmids specifying B-lactamases have been found in many strains of S. aureus which have been isolated more recently (Silvtr, 1978) .

Karmaker et al. (1991), examined isolates of Salmonella typhi from cases of typhoid during the 1989-1990 epidemic in Calcutta. Most isolates (84% of all isolates in the epidemic) were resistant to chloramphenicol, ampcillin, tetracycline and streptomycin but were sensitive to nalidixic acid. Plasmids of 120 Kilo-base and 14 Kilo-base were identified amongst the multidrug resistant isolates of

S. typhi . However there was no plasmid in the antibiotic sensitive isolates.

Drug resistance can also be specified by chromosomal genes, but the mechanism of resistance is uaually different to that specified by plasmids. Only chromosomal resistance to nalidoxic acid has been found so far (Kimber, 1981).

## Virulence Plasmids :

Pathogenic bacteria are able to withstand host-defence mechanisms and often produce toxins which damage the host. Both of these properties are sometimes plasmid-specified (Kimber, 1981).

E. coli strains can cause a variety of different types of disease. The most common in man are urinary tract infections, but E. coli is also a major cause of diarrhoea. At least three types of strain causing diarrhoea can be distinguished . Some strains cause a disease resembling dysentry which is caused by Shigella, epithelial cells of the colon are invaded and destroyed so that the faeces become mixed with blood. Other strains cause diarrhoea with little invasion of mucosa. The enterotoxigenic strains fall into this category. Many of these strains harbor plasmids which code for one or more types of enterotoxin. Enterotoxigenic strains are a common cause of acute diarrhoea in young, and in adults can cause traveller's diarrhoea which affects may visitors to tropical countries. A third class cause diarrhoea without extensive

tissue invasion , but do not produce detectable enterotoxins (Chakrabarty, 1976) .

Enterotoxigenic strains do not achieve their full pathogenic potential unless they are also able to colonize the small intestine effectively. The colonization factors which are commonly found on enterotoxigenic strains are protein pili which stick the bacteria onto the wall of the small intestine. These adhesive pili are often specified by plasmids which differ from plasmids code for enterotoxins (Drummond, 1979) .

E. coli strains can cause generalized infections among claves and chicks , they invade the body of the animal and occur in large numbers in the blood and various organs. In severe cases, death results from general septicaemia. Strains of E. coli responsible for generalized infection are an important cause of deaths among these animals. Particular serotypes are usually responsible for these infections, usually harbour plasmids which enhance their virulence. This was discovered by Smith (1976) who found that most of the strains causing generalized infection in these animals had special plasmids which increased bacterial resistance to host-defence mechanisms and produced and approximately 100 fold reduction in the lethal dose of E. coli for chicks (Kimber, 1981).

Certain strains of Staphylococcus aureus produce exfoliative toxin and can cause scalded-skin syndrome in susceptible people, mainly a young children . The skin

become loosened and may evently peel off in large sheets. Although the infection is initially confined to the skin around the mouth and nose, it can rapidly spread to most parts of the body. Production of exfoliative toxin by several S. aureus strains depend on the presence of a plasmid which encode the protein. Exfoliative toxin is psecified by chromosomal genes is some strains. (Calos and Miller, 1980) .

There are indications that factors which contribute to the invasiveness of Yersinia Pestis, the cause of bubonic and Pneumonic plague, are plasmid specified. A virulent variants of this bacterium (plasmid less cells) are readily isolated and usually lake several virulence determinants, including the ability to produce coagulase and fibrinolysin. The ability to produce bacteriocin is lost along with virulence determinants, suggesting that they are specified by the same plasmid (Kenneth et al., 1986).

The name bacteriocin was introduced by Jacob et al. (1953) as general term to define a class of antibiotic like substances produced by various species of bacteria and distinguished from other antibiotics by their limited range of action and their chemical nature (Chakrabarty, 1976).

Bacteriocins are active only against species of bacteria closely related to the strain producing them. They required a specific receptor site on the sensitive strain for their adsorption and killing action (Drummond, 1979).

Colicins are bacteriocins produced by E. coli and closely related members of Enterobacteriaceae such as Shigella sonnei; they are bactericidal for many enterobacteria. Substances analogous to colicins are produced by many Gram-positive and Gram-negative genera . For example, some Streptococci produce streptococcins and staphylococcins are produced by certain strains of Staphylococcus. in each case, the active component of bacteriocin is protein (Chakrabarty, 1976). Colicins are specified by Col plasmids, several staphylococcins and streptococcins are known to be plasmid encoded (Kimber, 1981). Also Col plasmids confer immunity to colicins they encode (Kenneth et al., 1986) .

**Metabolic Plasmids** :

The genus Pseudomonas includes bacteria which can grow on a very wide range of organic compounds. They play an important role in recycling carbon in the biosphere by degrading complex organic compounds to simpler forms which can be used as sources of carbon and energy by living organisms. Many of the degradative enzymes of Pseudomonas are specified by chromosomal genes, but it has recently become clear that plasmid-encoded enzymes are often involved (Chakrabarty, 1976) .

Degradative plasmids enable Pseudomonas Putida and related bacteria to grow on substrates such as, xylene, octane, camphor, naphthalene, salicylate and nicotinic acid. Plasmid encoded enzymes convert these growth substrates to metabolites such as acetaldehyde, pyruvate and acetate which can then enter metabolic pathways catalysed by chromosomal enzymes. Most degradative plasmids code for at least 10 enzymes which are involved in the catabolism of a particular substrate (Chakrabarty, 1976) .

Degradative plasmids enable bacteria to grow on synthetic compounds and may therefore make an important contribution to the removal of substances which can act as pollutants (Kimber, 1981).

Attempts are being made in several laboratories to use the metabolic potential of degradative plasmids by constructing strains for pollution control and for chemical synthesis (Kimber, 1981) .

Degradative plasmids are usually conjugative and have high molecular weights ranging from about $50 \times 10^6$ to about $200 \times 10^6$ daltons (Drummond, 1979).

Metabolic plasmids sometimes become apparent because they confer the ability to ferment unusual substrates so that strains give unexpected results when being identified or classified. Strains of Salmonella are almost always $lac^-$, that is, they are unable to ferment lactose. $lac^+$ strains of Salmonella typhi have been isolated on rare occasions and have been found to harbor plasmids which confer the $lac^+$ phenotype. Lac+ plasmids have also been found in strains

of Serratia and Proteus. Plasmids can also confer the usual ability to ferment sucrose on strains of Salmonella. So metabolic plasmids can occasionally lead to misidentification or to delays during bacteriological diagnosis of infectious diseases (Willetts and Skurray, 1980).

Curing Of The Plasmids :

One of the most common features of plasmids is that they can be eliminated from host cells by various treatments. This process termed "curing" apparently results from inhibition of plasmid replication without parallel inhibition of chromosome replication, and as the result of cell division the plasmid is diluted out (Tomoeda et al., 1983) .

Curing may occur spontaneously, but it is greatly increased by use of intercalating dyes such as acridine orange, acriflavine or ethidium bromide (Kado, 1977), as well as by other treatments that affect DNA replication. Such treatments may include , ultraviolet,ionizing radiation, heavy metals as well as growth at elevated temperature (Tomoeda et al., 1983) .

Plasmid molecules, which exist as autonomously replicating circular DNA duplexes ; are eliminated by these agents either because of interference with their replication (acridine and ethidium bromide) or by alteration of their

membrane attachment sites (sodium dodecyl sulfate (SDS) and elevated temperature) (Novick , 1969) .

Such effects on plasmids were first demonstrated by Hirota and Ijima (1957), who found that acriflavine eliminated the F factor from $F^+$ cells of E. coli and thereafter by Watanabe and Fukasawa (1961) , who reported that acriflavine and acridine orange eliminated certain resistance determinants from R. factors of multiple drug resistance bacteria. It was shown five years later that R. Factors are composed of DNA (Rownad et al., 1966).

Hahan and Clake (1971), reported that ethidium bromide and acridine orange eliminated the resistance determinats for kanamycin, chloramphenicol and ampcillin from on R Factors in E. coli chloroquine and quinine exhibited the same activity while methylene blue was non active.

Tomoeda et al. (1983), tested 16 strains of E. coli for elimination of antibiotic resistance markers of R. plasmid by subculturing E. coli strains in the presence of ethidium bromide and acriflavine, or at maximum temperature of 41°C. Partial and/or complete loss of resistance markers were observed with the frequency range of 20.8-44, 3.5-44 and 3.5-22.2% respectively. Such successful "curing" accured in 7 out of 16 tested strains. Consequently, it is concluded that drug resistance in these 7 host strains is controlled by R.plasmids.

Paul et al. (1987), in their studyof virulence plasmid in Salmonella gallinarum, using high temperature by growth of the organism at 42°C for 18 hours as a method for "curing" the plasmids. Loss of the small plasmid under these conditions was relatively common, occuring in approximately 1% of cells. The frequency of curing of the large plasmid was, however , much lower, accuring in less than 0.1% of cells. Plasmid loss was confirmed plasmid analysis .

Woodward et al. (1989), study the distribution of virulence plasmid with Salmonella, and in order to make a plasmid-free S. dublin, the plasmid of the derivative was tagged with transposon coding for Kanamycin resistance. Curing of the tagged plasmid was done by SDS and acridine orange treatment, then searching for kanamycin-sensitive clones by replica plating .

# SALMONELLAE

## Genus Salmonella :

William Budd in (1856), studied typhoid outbreaks and believed that typhoid was contagious and infectious agent, which was excreted in the faeces of the patients. He believed that contaminated water and milk played a role in the spread of the disease. Then Eberth in (1880), described typhoid bacillus in the tissues of the patient dying from typhoid fever. Later Gaffky in (1884), isolated the organism and subsequently Salmonella types were discovered and described by various workers (Chopra, 1985) .

## Nomenclature :

Nomenclature with the genus is complex, originally the Salmonellae were given species names that were descriptive of the disease they caused, such as S. typhi and S. enteritidis. Later as more antigenic types were described, a system of nomenclature was developed that named each new antigenic type according to geographical area where it was isolated. Therefore we have names such as S.panama and S. dublin. Similarly, they were named after the person who discovered them eg. S. schottmuelleri, or after the name of the person from whom the first strain was isolated eg S. thompson (Volk and Wheeler, 1984).

Kauffmann (1969), divided the Salmonellae into four biochemical groups or subgenera, the first of these inculdes the typhoid and paratyphoid bacilli and most of the other types that are responsible for wide spread disease in mammals, the remaining three subgenera comprise organisms that are the main parasites of cold blooded animals.

Ewing (1986), the concept has now evolved primarly through the work of International Subcomittee on Toxonomy of Enterobacteriaceae based on DNA relatedness studies, such that there is only one Salmonellae (S. choleraesuis) Most investigators have substituted the epithet (enterica) rather than (choleraesuis) because it avoids the use of a name that designates both a species and subtype

1. Salmonella enterica subspecies enterica.
2. Salmonella enterica subspecies salamae
3a. Salmonella enterica subspecies arizonae.
3b. Salmonella enterica subspecies diarizonae.
4. Salmonella enterica subspecies houtenae
5. Salmonella enterica subspecies bongori.

Although this nomenclature may be genetically valid, the toxonomy will be foreign to most clinical microbiologist and physicians. Within this nomenclature, the familiar terms, such as S.typhi and S. typhimurium, which related to established clinical syndromes, are lost. Therefore, on basis of clinical applications, the clinical laboratories are reporting organisms as serotypes such as salmonella serotype typhimurium, rather than using the complicated but

toxonomically correct Salmonella enterica subspecies enterica serotype typhimurium (Ewing, 1986) .

**Morphology** :

Salmonella bacilli are members of Enterobacteriaceae groups of organisms. They are Gram negative rods, non sporing, non capsulated and non acid fast, with parallel sides and rounded ends. About 2-4 microns in length and 0.5 micron in breadth (Noel, 1983).

Most species are motile with peritrichous flagella, except S. gallinarium and its variant pullorum . Non motile variants of normally motile organisms may be found under certain cultural conditions in the laboratory (Topley and Wilson's, 1984) .

Most Salmonellae are fimbriate (Volk and Wheeler 1984 ).

**Cultural Characters** :

The organisms are aerobic and facultatively anaerobic. Grow on simple laboratory media in the temperature range of 15-45°C, optimally at 37°C (Baker and Breach, 1980).

On peptone water and nutrient broth, most strains give abundant growth with uniform turbidity. A thin surface pellicle usually forms on prolonged incubation (topley and Wilson's 1990).

On nutrient and blood agar, the colonies are fairly large, with an average diameter of 2-3 mm, they may be circular and low convex with a smooth surface and entire edge, they may be flatter with a less regular surface and a more effuse serrated edge (Topley and Wilson's, 1984).

More often the mucoid appearance develops as a secondary phenomenon after prolonged incubation. Thus, when an agar plate is incubated in three or four places with the point of a needle, and after one day's incubation at 37°C is left at room temperature for few days, a large colonies are formed characterized by a depressed centre and surrounded by luxuriant mucoid wall (Chopra, 1985).

On MacConkey's medium, Salmonella are non lactose fermenters, thus produce after 18-24 hours at 37°C pàle yellow or nearly colourless, 1-3 mm in diameter and easily distinguished from the pink-red colonies of lactose-fermenting commensal coliform bacilli eg E.Coli which also grow well on this differential medium (Neol, 1983).

On deoxycholate-citrate agar (DCA), the colonies of Salmonellae are similar to or slighly smaller in size than those on MacConkey agar. They are pale, nearly colourless, smooth, shiny and translucent and easily distinguished from opaque pink colonies of lactose-fermenting coliform bacilli, which are largely inhibited on this selective differential medium (Volk and Wheeler, 1984) .

The selenite F and tetrathionate medium, inhibit the growth of coliform and other intestinal opportunists and thus may give pure growth of salmonella and so considered as enrichment media for them (Chopra, 1985).

Biochemical Reactions :

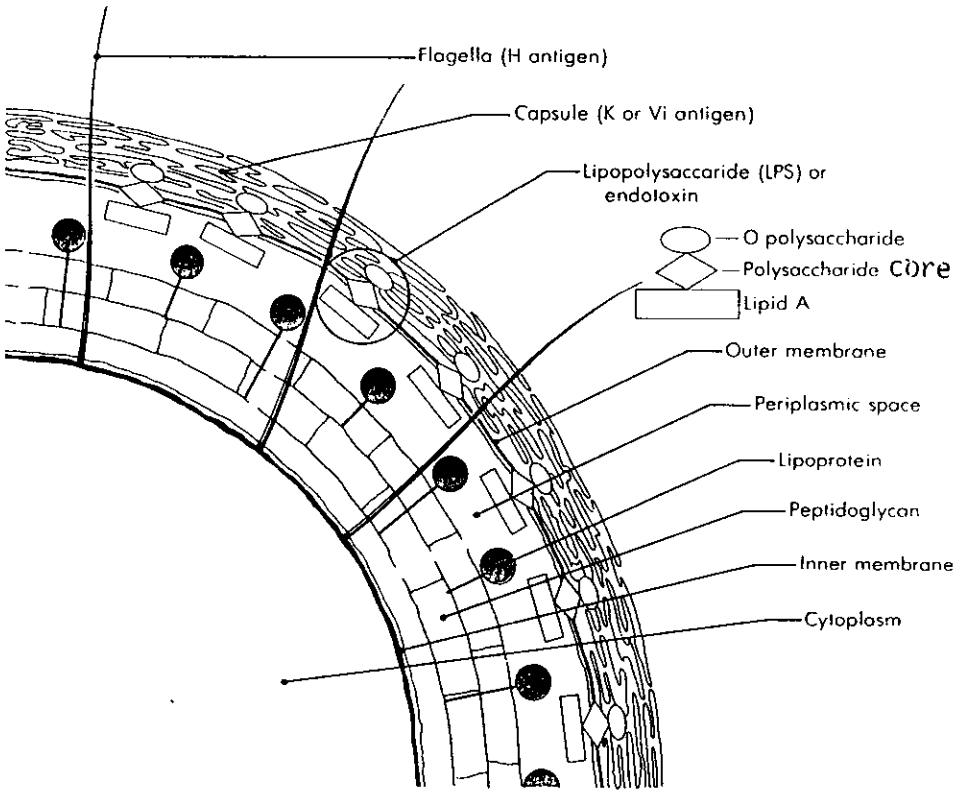
Salmonellae ferment certain carbohydrates with production of acid and gas, except in S.typhi which produce acid only and seldom gas. They are non lactose non sucrose formenter but glucose, maltose and mannite fermenters (Cheesbrough, 1991) .

They are catalase positve, oxidase negative, and reduce nitrate to nitrite, the organisms do not hydrolyse urea. Most Salmonellae also produce hydrogen sulphide in ferrous chloride gelatin medium except by S.paratyphi A. (Cheesbrough, 1991) .

They do not form indole or liquify gelatin (Noel, 1983).

The positive reaction to methyl-red test and the negative reaction to Voges-Proskauer test appear to be constant, while other biochemical characters are variable (Topley and Wilson's, 1990) .

Figure (3) : Antigenic structure of Enterobacteriaceae




(Murray and Thompson, 1990) .

Antigenic Structure :

The antigenic structure of Salmonellae is basically simillar to that of other Enterobacteriaceae, with two major kinds of antigens present : somatic (O) antigens and flagellar (H) antigens.

The O antigens are lipo-polysaccharide (LPS) of the outer membrane. These heat stable antigens own their antigenic specificity to the O-specific polysaccharides of the smooth lipo-polysaccharides (Volk and Wheeler, 1984).

The polysaccharide moiety contains the antigenic determinants, whereas the lipid moiety is responsible for endotoxin effect (Baker and Breach, 1980).

The structure of the lipo-polysaccharides is divided into three regions, Region I : contains the antigenic O-specific polysaccharide units which vary widely among different strains. Region II : contains an oligosaccharide "common core" shared among many different strains. Region III is the lipid part called lipid A (Figure 3 ) (Baker and Breach, 1980) .

The O antigen is less immunogenic than H-antigen and the titre of O-antibody after infection or immunization is generally lower than of H-antigens, which is strongly antigenic and induces antibody formation rapidly and in high titre following infection or immunization (Chopra, 1985).

The Flagellar (H) antigens are the proteins that make up the peritrichous flagella of these bacteria. In contrast to the O-antigens, the H-antigens are labile to heat and to treatment with ethanol and acid (Baker and Breach, 1980).

The H antigens of Salmonellae are diphasic, i.e., they can exist in either of two major phases- phase 1 or specific phase, and phase 2 or non-specific phase. The phase 1 antigens are shared by only a few organisms and react only with homologous antisera, whereas, the phase 2 antigens are shared by many organisms and will cross react with heterologous antisera (Topley and Wilsons, 1990).

A third antigen (Vi-antigen) is found in a few strains occuring as a heat-labile polysaccharide surface antigen, superficial to the O-antigens . Since it blocks agglutination by O-antisera, agglutinability in O-antiserum may be restored when this Vi-antigens is destroyed by heating (Germanier and Feur, 1975) .

M-antigen, which is a loose extracellular polysaccharide slime consisting of colanic acid. it occurs in a serologically similar form in various unrelated enterobacteria, including S.paratyphi B and many strains of E.coli Salmonellae form it most abundantly when, after for 1 day at 37°C, plate cultures are held for several days at ambient temperature, on such plates it causes the colonies to become mucoid, particularly at their margins, which then appear as a slime wall . it resembles the Vi antigen in preventing agglutination by O antibodies. heating for 2-5 hours renders the bacteria agglutinable by O antiserum

(Ewing, 1986).

There is also fimbrial antigens, which are formed by most strains of Salmonellae, bear antigens that determine agglutination by sera containing anti-fimbrial antibodies. The bacteria vary reversibly between a fimbriate phase, which predominates in 24-48 hours broth culture, and a non-fimbriate phase which predominates in young (6-hours) broth culture (Topley and Wilson's, 1990).

Classification :

White (1926) was the first who put the basis upon which the classification of Salmonellae was placed, then Kauffmann, confirmed, modified and greatly extended white's work and put the scheme of the present classification (Topley and Wilson's, 1984) .

In Kauffmann-white classification, Salmonellae divided into primary somatic groups, designated by letter A to Z and those discovered later after exhaustion of the alphabet, by the numbers (50, 51, 52, etc) Each group contains a number of serotypes possessing a common O-antigen not found in other O groups. Some groups are divided into subgroups whose members are distinguished by a second O antigens, e.g. group $C_1$ is characterized by O antigens 6 and 7, and group $C_2$ by O-antigens 6 and 8. The somatic antigens denoted by arabic numerals.

Subdivision of the major O-groups into serotypes was then accomplished by determination of the remaining O and H antigens, both phase 1 and phase 2, where phase 1 denominated

Table (1) : Kauffman-white classification of some Salmonellae of medical importance in developing countries.

| Salmonella | O Antigens | H antigens | |
|---|---|---|---|
| | | Phase I | Phase II |
| **Group A :** | | | |
| S.paratyphi A | 1,2,12 | a | - |
| **Group B :** | | | |
| S.paratyphi B | 1,4,5,12 | b | 1,2 |
| S.derby | 1,4,5,12 | f,g | (1,2)* |
| S.typhimurium | 1,4,5,12 | i | 1,2 |
| S.heidelberg | (1)*,4,5,(12)* | r | 1,2 |
| **Group $C_1$ :** | | | |
| S.cholerae-suis | 6,7 | c | 1,5 |
| S.paratyphi C | 6,7 (Vi)* | c | 1,5 |
| S.oranienburg | 6,7 | m,t | - |
| S.garoli | 6,7 | i | 1,6 |
| S.thompson | 6,7 | k | 1,5 |
| S.bareilly | 6,7 | y | 1,5 |
| **Group $D_1$ :** | | | |
| S.typhi | 9,12 (Vi)* | d | - |
| S.enteritidis | 1,9,12 | g,m | - |
| S.pullorum-gallinarum | 1,9,12 | - (non-motile) | - |
| **Group $E_1$ :** | | | |
| S.weltevreden | 3,10 | r | $z_6$ |
| S.anatum | 3,10 | e,h | 1,6 |
| **Group $G_2$ :** | | | |
| S.durham | 13,23 | b | $enz_{15}$ |
| S.worthington | 1,13,23 | z | 1,w |
| S.cubana | 1,13,23 | z 29 | - |

* Brackets indicate that the antigen may be present or absent.

(Cheesbrough, 1991).

by small letters, while those of phase 2 are accorded in two different ways. At first they have accorded arabic numerals, but later it was found that the second phase of certain serotypes contained the antigens e, n and x or z . Therefore, phase 2 may include antigenic components of the 1,2,3 series or the e, n, x series (Topley and Wilson's, 1984) .

In some serotypes, phase 2 contains neither 1,2,3 series nor the e, n, x series, such strains are monophasic serotypes and are characterized by phase 1 (Baker and Breach, 1980) (table 1).

So the antigenic formula for any serotype consists of three parts describing the somatic O-antigens denoted by arabic numerals, the phase 1 H-antigens by small Roman letters and the phase 2 H- antigens denominated by arabic numerals. Fore example the antigenic formula of Salmonella typhimurium is written as follows :

1,4,5,12 : i : 1,2 representing O antigens 1,4,5,12 : phase 1 H antigen i : phase 2 H antigen 1,2 .

Some microbiologists, however, prefer to use a modified Kauffmann-white terminology system. In the modified system , only the species S.typhi, S. cholerae-suis and S. enteritidis are included and all other Salmenellae are classified as serovars (ser) or bioserovars (bioser) of Salmonella enteritidis. The antigenic composition is the same for both classification. Examples of the unmodified and modified terminology systems are as follows :

infection of hurmans (Michael and Chan, 1981) .

Effect Of Physical And Chemical Agents On Salmonellae :

Salmonellae are readily killed by heat in 1-hour at 55°C and in 15 to 20 minutes at 60°C . They are destroyed by pasteurization temperatures in milk (Topley and Wilson's, 1984) .

Baking can be expected to kill all Salmonellae even when liquid egg used is highly contaminated (Nye and Roberts, 1979) .

Although Salmonellae are killed by temperatures readily reached and exceeded in cooking, the degree of heat penetration of the food is of the greatest importance for, though lethal temperatures may be attained on the surface of food, the temperature in the centre may be well below this (Bryan and Kilpatrick, 1971).

S.dublin could survive gentle frying for as long as 15 minutes although the sausages appeared well cooked (Pether and Scott, 1982) .

At low temperatures Salmonellae are not killed, but they do not multiply. The critical temperature appears to be above 5°C, below this temperature, salmonellae do not multiply, above it , they begin to multiply and spread, which vary with the nature of the infected food(Angelotti et al., 1981).

Another method of sterilization is gamma radiation of food stuffs, this method has been used experimentally with eggs, frozen meat and coconut. The radiation causes a negligable rise in temperature so that it can be used on frozen food. Another advantage is that food can be treated while still in its container (Bowmer, 1964).

Salmonellae can be destroyed by a wide range of chemicals including phenols, mercuric chloride, formaldehyde and ammonium compounds and any of these can be used for disinfecting surfaces and utensils (Bowmer, 1964) .

Chlorine and Potassium permanganate can be used for treating food, thus lettuce can be free from Salmonellae in 30 seconds by washing in water containing 80 parts per million chlorine (Nye and Roberts, 1979) .

**Pathogensis** :

After the organisms have been swallowed they are destroyed by the acid conditions in the stomach. If the number of the swallowed is small, they may all killed and non get through it to cause disease, but if the number is large, some may reach the intestine a live (Michael and Chan, 1981)

Milk and ice cream tend to neutralize gastric acidity and then allow even a small dose of salmonellae to survive and get through the small intestine (Noel, 1983).

Perhaps the speed with which the stomach empties is important, if the Salmonellae are swallowed in water they can propably pass through the stomach very quickly, where as, if in food they may held up and be destroyed (Duguid et al., 1966).

If the patient is taking antiacids for any reasons this might help to protect the Salmonellae in their passage through the stomach, and it seems to be true that patients with achlorhydria or patients who have had part of their stomach surgically removed are more liable than normal persons to suffer from salmonellosis (Gianella et al., 1970).

Having survived the passage through the stomach, Salmonellae establish themselves in the small intestine and are adsorbed to the intestinal epithelial cells. Intestinal motility or peristalsis tends to prevent this adsorption, so Dupont and Hornick (1973), reported that the use of antiperistaltic drugs, such as lomotil, in human enteritis can make the condition worse or lead to systemic invasion.

If Salmonellae are not all swept away by peristaltic action, they adsorb to intestinal epithelial cells of the small intestine and colon, at least in experimental animals, they penetrate these cells, as can be seen in electron microscopy studies and migrate through them and are extruded in-to lamina propria where they cause an inflammatory response with polymorphonuclears in most cases, but with macrophages with S.typhi. Where they are carried in these macrophages, invade the body and settles down to multiply

in the reticuloendothelial system . The others which cause gastroenteritis remain in the intestinal wall causing varying degrees of inflammatory reaction (Takeuchi, 1967).

In few cases Salmonellae invade the wall of the colon causing severe dysenteric symptoms with blood and mucus in the stool. Proctoscopy may show oedema and swelling of the mucosa and area of haemorrhage (Duguid et al., 1966).

S. Typhimurium 1,4 (5), 12 :i: 1,2 .

This organism was isolated in 1892 by Loeffler from rodents suffering from a typhoid like disease. Other specific names given to it include psittacosis and pestis caviae, it was frequently refered to in German literature as the "Breslau bacillus". Strains in which the Oantigen 5 is missing are somtimes referred to as the copenhagen (Topley and Wilson's, 1984) .

S.typhimurium usually give rise to an acute enteritis, but occasionally causes prolonged fever of the enteric type. It is probably the Salmonella with the widest distribution in the animal kingdam including man (Topley and Wilson's, 1984).

Buxton (1957), Lists 37 animal species from which it has been isolated. The part it plays in causing disease in the various species varies from country to country.

It was for many years common in cattle in Germany, and was the predominant Salmonella in cattle, sheep and horses in New Zealand (Josland, 1950).

In other countries however, antibiotic resistant strains for which an animal source could not be established have caused extensive epidemics in man (Topley and Wilson's, 1990) .

The effects produced by adminstration of living cultures of these organisms to small laboratory animals are entirely different from those produced by the typhoid bacillus, where we are dealing with the natural pathogen of these animals which give rise in them to a characteristic disease, usually known as mouse typhoid. This disease is produced when living cultures of S.typhimurium are given by the mouth as well as when they are adminstered by infection (Duguid and Campbell, 1969) .

Mice dying with 2-3 days after the injection of a moderate dose of a virulent strain will be found to have an acute speticaemia but mice dying after the more usual period of 5 to 10 days often show characteristic lesions, including a varying degree of splenic enlargment, with presence of small necrotic foci, larger and very characteristic necrotic lesions in the liver, and sometimes scattered pneumonic patches in the lungs, accompanied by a scanty pleural exudate (Seiffert et al., 1928) .

Salmonellosis :

Salmonellosis refers to infections caused by Salmonellae species, including acute enterocolitis, bacteriaemia, localized infection, typhoid fever, or paratyphoid fever (Noel, 1983)

The most common cause of non typhoidal salmonellosis in the United States is Salmonella enteritidis serotype typhimurium (Noel, 1983) .

Humans are infected by Salmonellas almost solely by the consumption of contaminated food or drink. Foods commonly responsible include cream containing pastries, sausages, commerically prepared beef roasts and eggs, the greastest source of salmonellosis is the reservoir of Salmonellas in lower animal (Michael and Chan, 1981).

Food outbreaks of salmonellosis are explosive in nature, and are associated with weddings, banquets and other events in which group meals are served. The suddenness of illness distinguishes it from other gastrointestinal diseases, such as bacillary and amebic dysentery (Michael and Chan, 1981) .

The incidence of salmonellosis varies with the season. The greast number of isolates are reported from July through October, the fewest are reported from December through May . This pattern is probably related to the opportunity given the microbes to multiply in food because of the warm temperatures prevailing from July through October (Baker and Breach, 1980) .

After contaminated food is ingested, the organisms multiply in the small intestine and colon. This leads to inflammation of the lamina propria of the villi. Penetration of the epithelial cells and migration to the lamina propria are prerequistes for causing disease. A distinguishing

clinical feature in the inflammatory response is that in cases of non typhoidal enterocolitis, the response is that of polymorphonuclear leukocytes, while in cases of typhoid and paratyphoid fevers, the response is a mononuclear one (Tokeuchi and Sprinz, 1967) .

Eight to forty-eight hours after eating food contaminated with Salmonellas, there is a sudden onset of abdominal pain and loose, watery diarrhoea, occasionally with mucus or blood. Nausea and vomiting are frequent, fever of 38°C to 39°C (100.4 to 102.2 F) is common (Michael and Chan, 1981) .

the mechanism where by these organisms induced diarrhoea remained a puzzle until the 1980s when Johnny Peterson and his Colleagues at the University of Texas demonstrated that lysed cultures of Salmonella form small amounts of a heat-labile enterotoxin similar to cholera enterotoxin. The reasons it has only recently been described appear to be two fold : (1) the toxin is synthesized in exceedingly small amounts and (2) the toxin does not appear to be readily released from the bacterial cell. Thus, it may not be functional until infecting organisms in the intestine spontaneously lyse to release the toxin into the intestinal environment (Volk and Wheeler, 1984).

Most non typhoidal Salmonellae infections follow a mild to moderate course. Serious illness are common in infants and in elderly patients with underlying disease (Noel, 1983) .

Children under ten years of age have the highest incidence of Salmonellae infection . The incidence is highest in children under one year, then drops off rapidly until about age ten (Noel, 1983) .

Definitive laboratory diagnosis of the disease depends on isolation of the bacteria from the feces. (Such bacteria should be the same as those isolated from the suspected food). Blood cultures are usually negative in Salmonella gastroenteritis. The use of selective or differential media such as MacConkey's agar medium is routine procedure. The identification of the microbes is then carried out by biochemical and serological methods (Michael and Chan, 1981) .

Antimicrobial agents are not recommended in the treatment of non-complicated nontyphoidal salmonellosis, because continuous administration of antimicrobials can lead to prolongation of the disease by fecal carrage of Salmonellae and appears to have no beneficial effect . Therapeutically it is of importance to correct dehydration and electrolyte disturbances. Therefore, attention should be focused on supportive and symptomatic therapy (Noel, 1983).

Severe manifestations due to non typhoidal Salmonellae obviously are indications for treatment with antimicrobial agents, but these are rare. Chloramphenicol is still the drug of choice in such diseases. Ampcillin is also a valuable drug in patients developing osteomyelitis due to non typhoidal Salmonellae if the strain is susceptible to

ampcillin. Ampcillin is very useful for treating patients with intravascular infections since chloramphenicol may not eradicate the bacteria from intravascular sites (Herzog, 1980).

There is no effective immunization against non typhoidal salmonellosis (Cherubin, 1973).

Little information is available on immunity to non typhoidal salmonellosis, but available data suggest that non typhoidal enterocolitis does not confer immunity. The same serotype of Salmonella has been shown to cause repeated attacks in individuals (Noel, 1983).

Measures that can be take against salmonellosis include : the proper cooking of foods obtained from animal source, such as meat and sausages, suitable refrigeration and covering of prepared foods; protecting food from contamination by mice, rats or flies and related insects, periodic inspection of food handlers and suitable sanitation.

Once a case of Salmonella food poisoning is discovered, it must be reported to public health authorities. This is extremely important, since suitable measures are called for in order to protect the public from an epidemic (Chopra, 1985) .

## VIRULENCE PLASMIDS AND SALMONELLAE

In 1970s several groups of scientists identified a large plasmid in strains of Salmonella typhimurium, however, with few exceptions, no phenotype could be ascribed to the plasmid. Hence, the plasmid was called a "cryptic plasmid". The cryptic plasmid was able to supress the fertility of the F plasmid and the plasmid could integrate into the chromosome of mutants of S.typhimurium to drive chromosomal replication (Sheehy et al., 1973).

Initially, Jones et al., in (1982), described the large cryptic plasmid of S.typhimurium as being associated with virulence. Since then, considerable work has been published concerning the pathogenesis associated with, and genetics of the high molecular weight vivulence plasmids of S.typhimurium and other serotypes and species of Salmonellae (Paul, 1990) .

Recent studies have indicated that large molecular mass plasmids are required for the virulence of several serotypes of Salmonellae which are capable of producing systemic diseases in animals and man. These include S.typhimurium, S. dublin, S.enteritidis, S.choleraesuis S.paratyphic and S.abortusovis (Barrow and Lovell, 1986).

Barrow and Lovell (1986), in their study on eight strains of S.pullorum isolated from epidemiologically in dependent cases of pullorum disease, otherwise (Known as bacillary white diarrhoea, in young and to lesser extent,

adult chickens, with considerable mortality and reduced egg production), found that at least one large molecular mass plasmid in addition to smaller molecular mass plasmid . The 85 kbp (Kilobase pair), large plasmid, designated pBL 001, of one of these strains was tagged with an ampcillin resistance marker by the insertion of transposon Tn3. The plasmid was eliminated by passage in nutrient broth containing acridine orange. It was reintroduced into strain from which it had been eleminated by mobilization using F plasmid. Following oral inoculation of newly hatched chickens, the natrual route of infection, the parent strain produce a high level of mortality (71%) with characteristic signs of pullorum disease. While the derivative lacking pBL 001 produced no mortality or morbidity when inoculated orally. Reintroduction of pBL001 restored virulence as gauged by oral inoculation of chickens. In addition, the parent strain, but not the pBL 001 cured derivative, localized in large numbers in the myocardium where it produced lesions typical of pullorum disease.

In another study, of two mutant strains of*S. dublin* which had substantially lost virulence through a single transposon insertion in the resident plasmid and comparison had been made of the behaviour of the parent and mutant strain in virulence experiments in mice. Authors found that *S. dublin* plasmid of mol.wt 46 x $10^6$ daltons is important for the pathogenesis of the organism where the virulence (expressed as lethal dose -LD50-values) for mice of two mutant strains,

was reduced by $10^4$-$10^5$ fold when infection was by the oral or intravenous or intraperitoneal route. However, the plasmid does not seen to affect the ability of the organism to cross the mucous gut wall, because loss of virulence in the mutants was unaffected by the route of inoculation. It seemed possible that their reduced virulence might be due to diminished ability to resist host defences. One aspect of resistance to host defences is the ability of certain bacteria to resist the bactericidal action of normal serum, which require further investigations (Jane et al., 1986) .

S. gallinarum produces fowl typhoid of poultry which is a disease of major economic importance in many countries. Unlike the closely related avain pathogen S. pullorum, S. gallinarum is able to produce disease in both young and adult chickens. Mortality associated with the disease is frequently high, under experimental conditions with newly hatched chickens it can be 100% (Smith and Tucker, 1980).

Paul and Co-worker (1987), in their study of four strains of S. gallinarum isolated from independent cases of fowl typhoid, found that all possessed both an 85 Kilobase and a 2.5 kilobase plasmid. Each plasmid was eliminated in turn from one of the strains by transposon tn3 labeling and curing at 42°C. Strains cured of each plasmid were identified by their sensitivity to ampcillin, resistance to which is encoded by Tn3. Elimination of the small plasmid had no effect on the high virulence of the strain for newly hatched and 2-weeks old chickens. Whereas oral inoculation of 2-weeks old chickens with the parent strain produced 90%

mortality with characteristic signs of fowl typhoid, inoculation of the large plasmid minus strain produced 0% mortality. Reintroduction of the large plasmid completely restored virulence. These results clearly demonstrate that the large plasmid of S. gallinarum contributes toward virulence in fowl typhoid of chickens.

To assess whether the large plasmid had a role in bacterial invasion, 3 weeks old chickens were inoculated orally with parent and large plasmid-minus strains and the presence of bacteria in the liver and spleen was monitored. In 3 weeks old chickens the parent strain persisted in the alimentary tract and was isolated from the liver and spleen by 60 hours post inoculation. In contrast, the large plasmid-minus strain was eliminated from the alimentary tract after 24 hours and was not isolated from the liver and spleen during the course of the experiment. Also to assess the viability of the strains in the reticuloendothelial system, both strains were inoculated intravenously into 3-weeks old birds, and the presence of bacteria in cardiac blood, liver and spleen samples was monitored. The large plasmid-minus strain was cleared from the blood by 2 days post inoculation. The viable counts of this strain in the liver and the spleen decreased rapidly and the chickens remained healthy. In contrast the counts of the parent strain increased in the blood, liver and spleen, until by 8 days post inoculation all birds were dead. These results suggested that the plasmid had a role in pathogenesis both

They also observed that only plasmid-psoitive strains could invade the livers of orally infected mice. and only they were resistant to the bactericidal activity of guinea pig serum. Strains of S. infants were generally plasmid free, whereas S. panama and S. heidelberg isolates carried heterogenous plasmid populations. The virulence properties of the latter two serotypes could not be correlated with the predominant plasmids found in these strains (Helmuth et al., 1985) .

Jones et al. (1982) found that removal of a 90 kbp plasmid harboured by several S. typhimurium isolates led to reduced virulence in mice and loss of ability to adhere to and invade hela cells. Reintroduction of the plasmid restored the adhesive and invasive phenotype.

Michiels et al. (1987), found a high correlation between the presence of a 90 kbp plasmid and virulence of S. typhimurium . After oral infection, both the plasmid-bearing and the plasmidless strains exhibited the same capacity to colonise coecal content, to invade Peyer's patches and to translocate to mesenteric lymph nodes in mouse model. However loss of the plasmid is correlated with an important reduction in the ability to colonise spleen and liver. Strain laking this plasmid was also shown to be more sensitive to bactericidal action of serum (Michiels et al., 1987).

Williamson et al. (1988), found that cured derivatives of S. typhimurium showed reduced virulence by $10^2$ fold following oral infection of mice.

Gulig and Curtiss (1988), had found that 100 kbp virulence plasmid of S. typhimurium is primarily responsible for conferning the potential for systemic infection from Peyer's patches to spleens of mice inoculated orally with S. typhimurium . They inoculated BALB/c mice 6 to 11 weeks old, orally after food and water deprivation and monitored for death up to 30 days postinoculation. Infected mice were housed in cages with raised wire floors and filter lids to prevent cross-contamination between cages. they also had found that curing of virulence plasmid had no effect on resistance of S. typhimurium to normal human serum.

In contrast, the invasiveness of S. typhimurium is thought to be chromosomally mediated (Hackett et al., 1986).

Relatively little is known about plasmid DNA sequences involved in S. typhimurium virulence expression. To localise the sequences involved in virulence on the 90 kbp , a new method based on deletions to generate strains carring various deletion mutations which were tested for virulence in mice. It was found that a large region of the plasmid could be deleted without affecting the virulence. The virulence determinants are thus clustered in a single region of the plasmid in contrast to the situation encountered in Yersinia where virulence determinants are scattered all over the plasmid (Michiels et al., 1987).

Soon after the discovery that a virulence plasmid existed in S. typhimurium, plasmids with DNA homology were found in other species and serotypes of Salmonellas. Paul in (1990), determined that large plasmids of S. typhimurium, S. enteritidis, S. paratyphic , S. newport and S. abortusovis were related at the level of DNA homology and restriction fragment mapping .

Genetic and physical analysis of these virulence plasmids had led to identifications of genes specifically involved in virulence. For those plasmids best studied from S. typhimurium, S. dublin and S. choleraesuis, a consensus region of 8 kbp- SalI-Xho I fragment is essential for full expression of virulence. Four virulence genes had been mapped on this regions. The different virulence plasmids share large regions of DNA homology as based on DNA hybridization studies (Montenegro et al., 1991).

Qu et al. (1990) , examined the compatibility of Salmonella virulence plasmids of serovars choleraesuis dublin, enteritidis, gallinarum and pullorum and cryptic Salmonella plasmids of serovars cophenhagen, durban, give, infantis and sendai, with the gokbp virulence plasmid of S. typhimurium, which was found to be incompatible with the plasmids of serovars choleraesuis, copenhagen, dublin, enteritidis and sendai but compatible with plasmids of serovars durban, gallinarum, give, infantis and pullorum.

So related high molecular weight plasmids of several serotypes and species of Salmonellas have been associated with virulence in a variety of animal models of infection. The primary virulence plasmid phenotype is in the ability of Salmonellae to spread beyond the initial site of infection, the intestine. The mechanism of this plasmid-mediated invasive infection has not been identified, but may be a complex interaction in the host pathogen relationship. A common region of Salmonella plasmids has been associated with virulence and specific virulence genes and their products are now being identified, however, much is yet to be accomplished in this field (Paul, 1990) .

# MATERIALS AND METHODS

## A. Bacterial Strain :

Salmonella typhimurium strain was obtained from Central laboratories of Ministry of Health, Cairo. The strain was examined by :

1. Gram stain film .

   It was Gram negative bacilli.

2. Culture :

   Culture on MacConkey agar at 37°C for 18 hours showing, pale colonies, 1-3 mm in diameters, smooth, circular with entire edge.

3. Biochemical tests :

   The strain fermented glucose, maltose and mannite with production of acid and gas, but lactose and sucrose was not fermented.

   Indole was not produced, methyl-red was positive . Acetyl methyl carbinol was not produced (i.e. Voges-Proskeuer-negative). Citrate was utilized but urease was not produced. hydrogen sulphide was produced and gelatin was not liquified.

4. Serodiagnosis :

   It produced agglutination by slide agglutination test against Salmonella polyvalent antisera.

5. Animal Pathogenicity (Michael and Chan, 1981).

   0.1 ml of the strain broth culture which incubated for 3 hours at 37°C was injected intraperitoneal of white mouse and the animal was died 24 hours after injection and the strain could be isolated from the heart blood, spleen and liver of the dead mouse .

B. **DNA Plasmid Curing** :

*** Material :**

- Broth medium and nutrient agar plates (Sigma USA).

*** Equipment :**

- Shaking water-bath
- Disposable microcentrifuge tubes
- Appropriate pipettes and petri dishes.
- Micropipettes .
- Glass spreader.
- Vortex .

*** Methods :**

1. Broth culture :

   One colony of Salmonella typhimurium strain was inoculated on 10 ml broth medium.

   The cutlure was incubated in shaking-water bath for 18 hours (overnight) at 42°C, aming at curing of the plasmid (if present) (Paul et al., 1987) .

2. Serial dilutions of the overnight broth culture medium were prepared as follows :

   Pipette 900 ul of sterile broth medium into each of several (8) sterile microcentrifuge tubes.

   100 ul of the overnight broth culture of the strain was mixed by vortex with 900 ul of broth present in the first tube.

   A ten fold serial dilutions was done, using micropipet where 100 ul of the previous mixture was transferred to the 2nd microcentrifuge lube (containing 900 ul broth) mixing well with vortex.

   Then 100 ul of the 2nd tube was transferred to the 3rd tube (which containing 900 ul of broth medium) and so on till the end of the 8 tubes.

   100 ul from each of the last three dilution tubes was spread onto a seperate, labelled, dry nutrient agar plate using a sterile glass spreader and incubated .

3. Twenty separate colonies from these plates were choosen, and each colony was added to 2 ml broth medium in a seperate microcentrifuge tube and allowed to incubate for 18 hours at 37°C.

4. These twenty seperate colonies were examined for their plasmid content by plasmid analysis to determine plasmid +ve strain (wild type) and plasmid -ve mutant.

C. Plamid Isolation (By Alkaline Lysis Method Of Ausubel et al., 1987) .

*Reagent :

1. Glucose/ Tris / EDTA (Ethylene-Diamine -Tetra-Acetic acid) - (GTE) solution .

   . Glucose 50 mM (mili-mole) (Sigma USA).

   . Tris-cl 25 mM . (Sigma USA).

   . EDTA 10 mM (Sigma USA).

   This solution was autoclaved and stored at 4°C .

2. NaOH (Sodium hydroxide) / SDS (Sodium dodecyl-sulphate).

   . Normal (N) NaOH 0.2 (Sigma USA).

   . SDS 1 % (Sigma USA).

   This solution was prepared immediately before use.

3. 5M potassium acetate solution, pH 4.8

   . 29.5 ml glacial acetic acid (Sigma USA)

   . KoH (potassium hydroxide) pellets to pH 4.8 (Several) (Sigma USA) .

   This solution stored at room temperature.

4. Ethanol 95 % and 70% (Sigma USA) .

5. DNase - free RNase 10 mg/ml (Boreingher Manheim, Germany) .

* Equipment :

- 1.5 ml disposable microcentrifuge tubes.
- Fixed angle cooling microcentrifuge .
- Vortex .
- Pasteur pipettes - micropipettes.

* Principles :

The alkaline lysis procedure is the most commonly used miniprep (Ausubel et al., 1987) . Plasmid DNA is prepared from small amounts of many different cultures (1-24) of plasmid containing bacteria. Bacteria are lysed by treatment with a solution containing SDS and NaOH, (SDS denatures bacterial proteins and NaOH denatures chromosomal and plasmid DNA) . The mixture is neutralized with potassium acetae, causing the covalently closed plasmid DNA to re anneal rapidly . Most of the chromosomal DNA and bacterial proteins precipitate as dose the SDS which form a complex with potassium and are removed by centrifugation. The reannealed plasmid DNA from the supernatant is then concentrated by ethanol precipitation (Ausubel et al., 1987).

Procedure :

1. 1.5 ml of broth culture ( from each of the previous 20 colonies after they were incubated overnight at 37°C) were allowed to spin for 20 seconds in a microcentriguge

at maximum speed (30,000 rpm) to pellet. Then the supernatant was removed with pasteur pipet.

2. The pellet was resuspended in 100 ul GTE solution and maintained for 5 min at room temperature.
3. 20 ul NaOH/SDS solution were added and mixed by tapping the tube with finger, and placed on ice for5 min.
4. 15 ul potassium acetate solution were added and mixed by vortex at maximum speed for 2 seconds and placed on ice for 5 min.
5. The samples were spined again as in step (1) to pellet cell debris and chromosomal DNA.
5. The supernatant was transfered to a fresh tube, mixed with 0.8 ml of 95% ethanol and kept at room temperature for 2 min to precipitate nucleic acids.
7. The samples were spined for 1 min at room temperature to pellet plasmid DNA and RNA
8. The supernatant was removed, and the pellet was washed by 1 ml of 70% ethanol and was dried under vaccum.
9. Contaminating RNA may interfere with detection of DNA fragments on the agarose gel, so to destroy them 1 ul of à 10 mg/ml RNase solution (DNase-free) was added to the digestion mixture.

## D. Resolution Of Plasmid DNA Fragment On Standard Agarose Gel :

*** Materials :**

. Electrophoresis buffer (Sigma USA).

. Ethidium bromide (Sigma USA).

. 10 X loading buffer (Sigma USA).

. DNA molecular weight marker (Boreinher Manheim, Germany).

*** Equipment :**

. 55°C water bath

. Horizonal gel electrophoresis apparatus* (designated by Dr. Roshdan Arafa).

. Electrophoresis-grade agarose (Sigma, USA).

. Gel casting platform.

. Gel coombs.

. Power supply.

---

* This apparatus was standerdized with original apparatus and give the same results.

*** Principle :**

Agarose gel electrophoresis is a simple and highly effective method for seperating, identifying, and purifying DNA fragments. The protocol can be divided into 3 stages : (1) a gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated, (2) the DNA are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation, and (3) the gel is stained or, if ethidium bromide has been incoprorated into the gel and electrophoresis buffer, visualized directly upon illumination with ultraviolet light (Ausubel et al., 1987).

*** Procedure :**

1. An adequate volume of electrophoresis buffer was prepared to fill the electrophoresis tank and to prepare the gel.
2. To facilitate visualization of DNA fragments during the run, ethidium bromide was added to electrophoresis buffer to a final concentration of 0.5 ug/ml.
3. 0.6 gram of electrophoresis grade agarose was added to 100 ml of electrophoresis buffer for constructing the gel.
4. The agarose was melted in microwave oven and cooled to 55°C in a water bath before pouring onto the gel platform to prevent warping of gel apparatus.

5. The gel casting platform was sealed at the ends and poured in the melted agarose at thickness between 0.5 and 1 cm.
6. The gel comb lastly inserted and made sure that no bubbles were trapped under neath the comb and all bubbles on the surface of the agarose were removed.
7. After the gel had hardened, the tap was removed from the open ends of the gel platform and also the gel comb was removed.
8. The gel casting platform containing the set gel was placed in the electrophoresis tank. And a sufficient electrophoresis buffer was added to cover the gel at a depth of about 1 ml and made sure that no air pockets were trapped within the wells.
9. Samples were typically loaded into wells with a micropipet after addition of appropriate amount of 10 X loading buffer.
10. Molecular weight marker was loaded into at least two wells with a micropipet on both sides of the samples.
11. Power supply was turned on to begin electrophoresis so that the DNA would migrate into the gel towards the anode or positive leads.
12. The progress of seperation could be monitored by migration of bromophenol blue dye in the loading buffer.

13. The power supply was turned off when the dye from the loading buffer had migrated a distance judged sufficient for seperation of DNA fragments.

## E. Visualization And Photography Of DNA In Agarose Gels :

1. Plasmid DNA was visualized by placing the agarose gel on ultraviolet illuminator which was typically used for this purpose - Protectiwe eye wear was warn at all times while using ultravialet (UV) light source, since this tight is damaging to the eye and exposed skin .
2. Plasmid DNA was photographed in agarose gels stained with ethidium bromide by illumination with ultraviolet light and Polaroid MP4 camera was equipped with Polaroid film holder provided a convenient means for gel photography.
3. Polaroid type 667 film was used which offered ideal sensitivity.

## F. Determination Of Plasmid +ve And Plasmid -ve Strains :

1. After we had chicked the presence of plasmid in the previous prepared 20 separate colonies of S. typhimurium strain by horizonal gel electrophoresis. At this step of work, at least one plasmid (+ve) (wild type-original type) and one plasmid (-ve) ( mutant type) could be determined.

2. From the original culture of the previously chosed two strains , plates were prepared and overnight broth culture was done for both strains.
3. A ten fold serial dilution was done for both strains (as disscussed in page 56).

## G. Bacterial Count (According to Chopra, 1985) :

1. The plates of the nutrient agar were dried at 37°C to be able to absorb all the water of the inoculum before the bacteria can multiply.
2. 100 ul of each dilution was taken and pipetted on to the surface of the 8 plates and at once was spreaded widely with sterile glass spreader.
3. The plates were incubated overnight (18 hours) at 37°C .
4. The colonies were counted and the number of the organisms could be determined per milliter of culture for both mutant and(original strain)or wild strain .

## H. Animal Experiment :

**. Brinciples :**

- Serial dilutions in saline for mutant strain (plasmid -ve) and wild strain (plasmid +ve) were inoculated orally in mice to test the virulence of the strain and the role of plasmid in this virulence. The 50% endpoints or LD50 was used to measure the virulence of both strains. It is the

end point in which half of the animals react or die and the other half do not (Nakamura et al., 1985) .

. **Procedure :**

1. 60 BALb/c mice of the same breed (4-6) weeks and about the same weight were obtained from the animal house of National Research Center, Cairo .
2. they were housed in special cages with proper aeriation and were supplied by special diet containing milk and vitamines specific for this species of mice.
3. Adequate ventilation from the windows, were ensured, but with great care to avoid exposure of the cages to draughts.
4. The cages were cleaned more frequent to avoid cross infection.
5. Groups of mice were inoculated orally with the serial dilutions of one of the strains whether the wild or the mutant one by special sterile cannula after food and water deprivation for 8 hours .
6. The mice were observed for 4 weeks and the number of deathes were calcualted and were burned every day.
7. the mice were divided into 15 groups, four mice for each group .

**Group (1)** : normal control group "non infected" inoculated by saline (which used for preparing the serial dilutions of the strains) .

**Group (2)[a]** : inoculated orally by 0.1 ml/mouse of concentration $1 \times 10^9$ cells / ml of plasmid +ve strain .

**Group (2)[b]** : inoculated orally by 0.1 ml/mouse of concentration $1 \times 10^9$ cells/ml of plasmid -ve strain .

**Group (3)[a]** : inoculated orally by 0.1 ml / mouse of concentration $1 \times 10^7$ cells / ml of plasmid +ve strain .

**Group (3)[b]** : inoculated orally by 0.1 ml/mouse of concentration $1 \times 10^7$ cells/ml of plasmid -ve strain .

**Group (4)[a])** : inoculated orally by 0.1 ml/mouse of concentration $1 \times 10^6$ cells/ml of plasmid +ve strain .

**Group (4)[b]** : inoculated orally by 0.1 ml/mouse of concentration $1 \times 10^6$ cells 1 ml of plasmid -ve strain .

Group $(5)^a$ : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^5$ cells / ml of plasmid +ve strain .

Group $(5)^b$ : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^5$ cells/ ml of plasmid -ve strain .

Group $(6)^a$ : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^4$ cells / ml of plasmid +ve strain .

Group $(6)^b$ : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^4$ cells / ml of plasmid -ve strain .

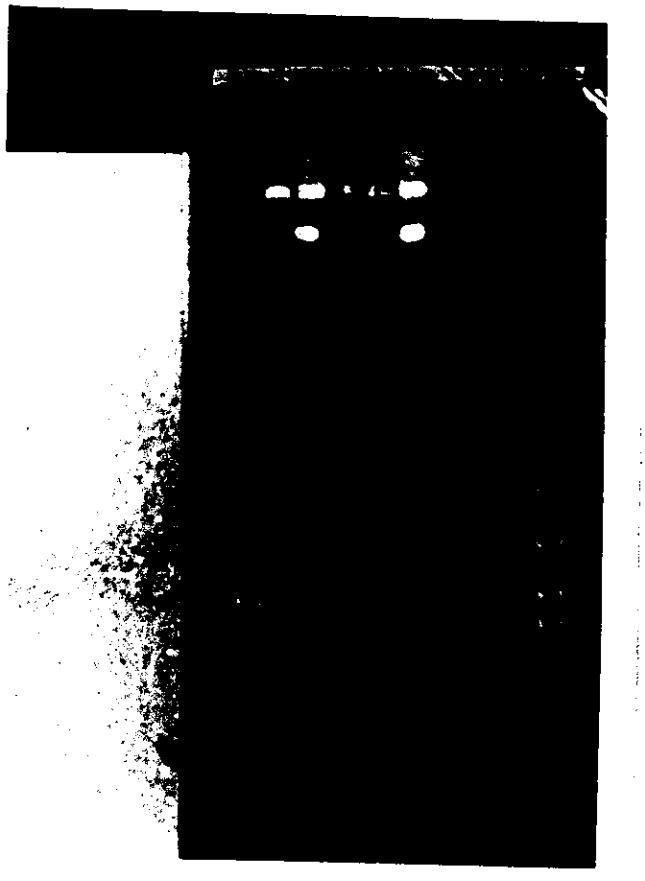
Group $(7)^a$ : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^3$ cells / ml of plasmid +ve strain .

Group $(7)^b$ : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^3$ cells/ ml of plasmid -ve strain .

**Group (8)[a]** : inoculated orally by 0.1 ml/ mouse of concentration 1 X $10^2$ cells / ml of plasmid +ve strain .

**Group (8)[b]** : inoculated orally by 0.1 ml/mouse of concentration 1 X $10^2$ cells/ ml of plasmid -ve strain .

**Figure (4)** : Submarine horizontal electrophoresis of S.typhimurium strain after plasmid isolation by alkaline lysis method.



Lane 1,7,10, represent the molecular weight marker

Lane 2,4,5,8,9 represent the plasmid -ve mutant

Lane 3,6 represent the plasmid +ve strain .

* **Virulence Test :**

C. The number of deaths in groups of mice whether control group or groups inoculated by plasmid +ve strain and groups inoculated by plasmid -ve strain was demonstrated in figures(5,6 ) and were as follows :

Control group : no deaths were recorded through the period of experiment (28 days).

Group $2^a$ : all mice died .

Group $3^a$ : all mice died.

Group $4^a$ : all mice died

Group $5^a$ : 3 mice died .

Group $6^a$ : 3 mice died

Group $7^a$ : 3 mice died

Group $8^a$ : 2 mice died

Group $2^b$ : all mice died

Group $3^b$ : 3 mice died

Group $4^b$ : 1 mice died

Group $5^b$ : no deaths

Group$6^b$ : no deaths

Group $7^b$ : no deaths

Group $8^b$ : no deaths .

D. The 50% end point or the 50% lethal dose value for both wild and mutant strain was measured by the method of Reed and Muench, 1938 .

**Computation of 50% Endpoints** :

In biological quantitation, the end-point is usually taken as the dilution at which a certain proportion of the test animals react or die. The most desirable end point is one in which half of the animals react and the other half do not. To avoid the costly use of large numbers of animals at many test dilution, Reed and and Muench devised a simple method for estimating 50% end points.

Their method is applicable primarily to complete titration series, ie., the whole reaction range, from 0% to 100% mortality or infectivity or cytopathic effect.

* Accumulated values for total number of animals that dided or survided are obtained by : adding in the directions indicated by arrows in column c and d (as demonstrated in table 2,3 ).

* The accumulated mortality ratio (coulmn g ) repreasents : the accumulated number of dead animals (coulmn e) over the accumulated total number .

Therefore, the 50% end point lies some where between dilution above 50% and that in the next lower dilution.

Since the distance between any two dilutions is a function of the incremental steps used in preparing the series eg. 2 fold, 5 fold, 10 fold, it is necessary to correct (multiply) the proportionate distance by the dilution factor, which is the logarithm of the dilution steps employed.

The necessary proportionate distance of the 50% mortality end-point, which obviously lies between the two dilutions, is obtained from column h as follows :

$$\frac{\text{\% mortality at dilution next above 50\% - 50\%}}{\text{\% mortality at dilution next above 50\% - \% mortality at dilution next below 50\%}} = \text{proportionate distance.}$$

Case of serial dilution-10 fold dilution, the factor is 1 (log 10=1) and so is disregarded, in 2 fold dilution series, the factor is 0.3 (log of 2), in a 5- fold series, the factor is 0.7 (log of 5) etc. In the procedures which follows, the factor is understood to be negative.

Therefore the negative log of LD50 ends points titre equals the negative log of dilution above 50% mortality pluse the proportionate distance X dilution factor (log 10) or (-1).

Table (2) : Shows the data of the wild form of S. typhimurium strain for measurement of LD50

| Bacterial Dilution(a) | Mortality ratio (b) | Died (c) | Survived (d) | Accumulated Values | | | |
|---|---|---|---|---|---|---|---|
| | | | | Total dead (e) | Total survived (f) | Mortality Ratio (g) | Mortality % (h) |
| $10^{-1}$ | 4/4 | 4 | 0 | 23 | 0 | 23/23 | 100% |
| $10^{-3}$ | 4/4 | 4 | 0 | 19 | 0 | 19/19 | 100% |
| $10^{-4}$ | 4/4 | 4 | 0 | 15 | 0 | 15/15 | 100% |
| $10^{-5}$ | 3/4 | 3 | 1 | 11 | 1 | 11/12 | 92% |
| $10^{-6}$ | 3/4 | 3 | 1 | 8 | 2 | 8/10 | 80% |
| $10^{-7}$ | 3/4 | 3 | 1 | 5 | 3 | 5/8 | 63% |
| $10^{-8}$ | 2/4 | 2 | 2 | 2 | 5 | 2/7 | 29% |

$$\frac{\text{\% mortality at dilution next above 50\% - 50 \%}}{\text{\% mortality at dilution next above 50\%-\% mortality at dilution next below 50\%}} = \text{Proportionate distance}$$

i.e. $\frac{63\ \% - 50\ \%}{63\ \% - 29\ \%} = 0.4$

Log of dilution next above 50 % = 7

* Negative log of dilution above 50% mortality + proportionate distance x dilution factor (-1) = negative log of LD50 .

i.e. $-7 + 0.4 \times -1 = -7 + -0.4$

$= -7.4$

i.e. LD50 titre of the wild form = 25118864

$= 3 \times 10^{-7}$

$= 3 \times 10^{3}$ C.F.U./ml

(Colony-Forming Unit/ml).

**Figure (5) : Shows the number of mice Killed by serial dilutions of the wild form of S. typhimurium strain in each group per day .**

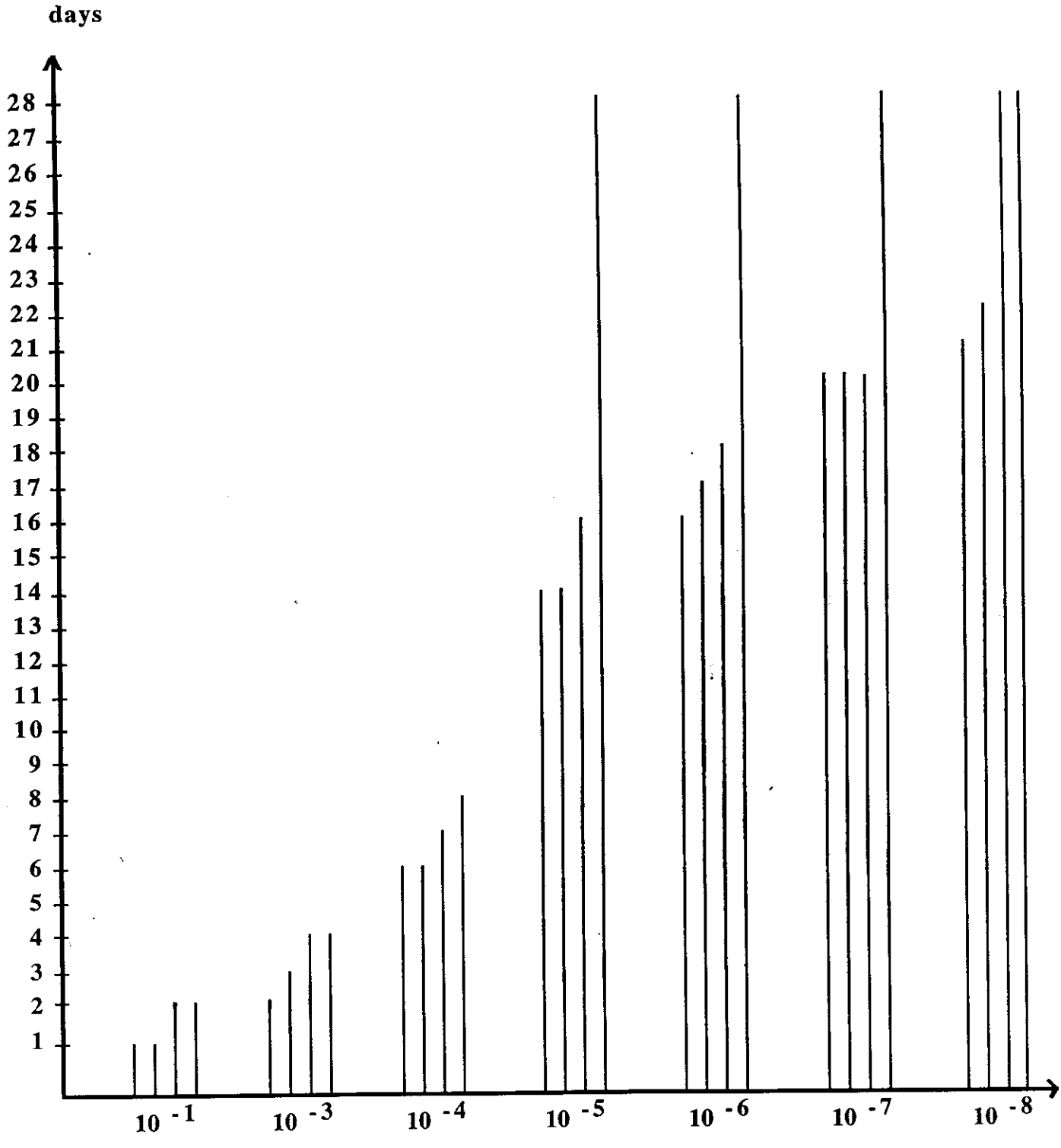


Table (3) : Show the data of the mutant form of S. typhimurium strain for measurement of LD50 .

| Bacterial dilution(a) | Mortality ratio (b) | Died (c) | Survived (d) | Accumulated Values | | | |
|---|---|---|---|---|---|---|---|
| | | | | Total dead (e) | Total survived(f) | Moratlaity Ratio (g) | Moratlaity %(h) |
| $10^{-1}$ | 4/4 | 4 | 0 | 8 | 0 | 8/8 | 100% |
| $10^{-3}$ | 3/4 | 3 | 1 | 4 | 1 | 4/5 | 80% |
| $10^{-4}$ | 1/4 | 1 | 3 | 1 | 4 | 1/5 | 20% |
| $10^{-5}$ | 0/4 | 0 | 4 | 0 | 8 | 0/8 | 0 % |
| $10^{-6}$ | 0/4 | 0 | 4 | 0 | 12 | 0/12 | 0 % |
| $10^{-7}$ | 0/4 | 0 | 4 | 0 | 16 | 0/16 | 0 % |
| $10^{-8}$ | 0/4 | 0 | 4 | 0 | 20 | 0/20 | 0 % |

$$\frac{\text{\% mortality at dilution next above 50\% - 50\%}}{\text{\% mortality at dilution next above 50\% - \% mortality at dilution next below 50 \%}} = \text{Proportionate distance}$$

i.e. $\frac{80\ \% - 50\ \%}{80\% - 20\%} = 0.5$

Log of dilution next above 50% mortality = 3

Negative log of dilution above 50% mortality + Proportionate distance X dilution factor (-1) = negative log of LD50

i.e. $-3 + 0.5 \times -1 = -3 + -0.5$

$= -3.5$

i.e. $LD_{50}$ titre of the mutant form $= -3162.27$

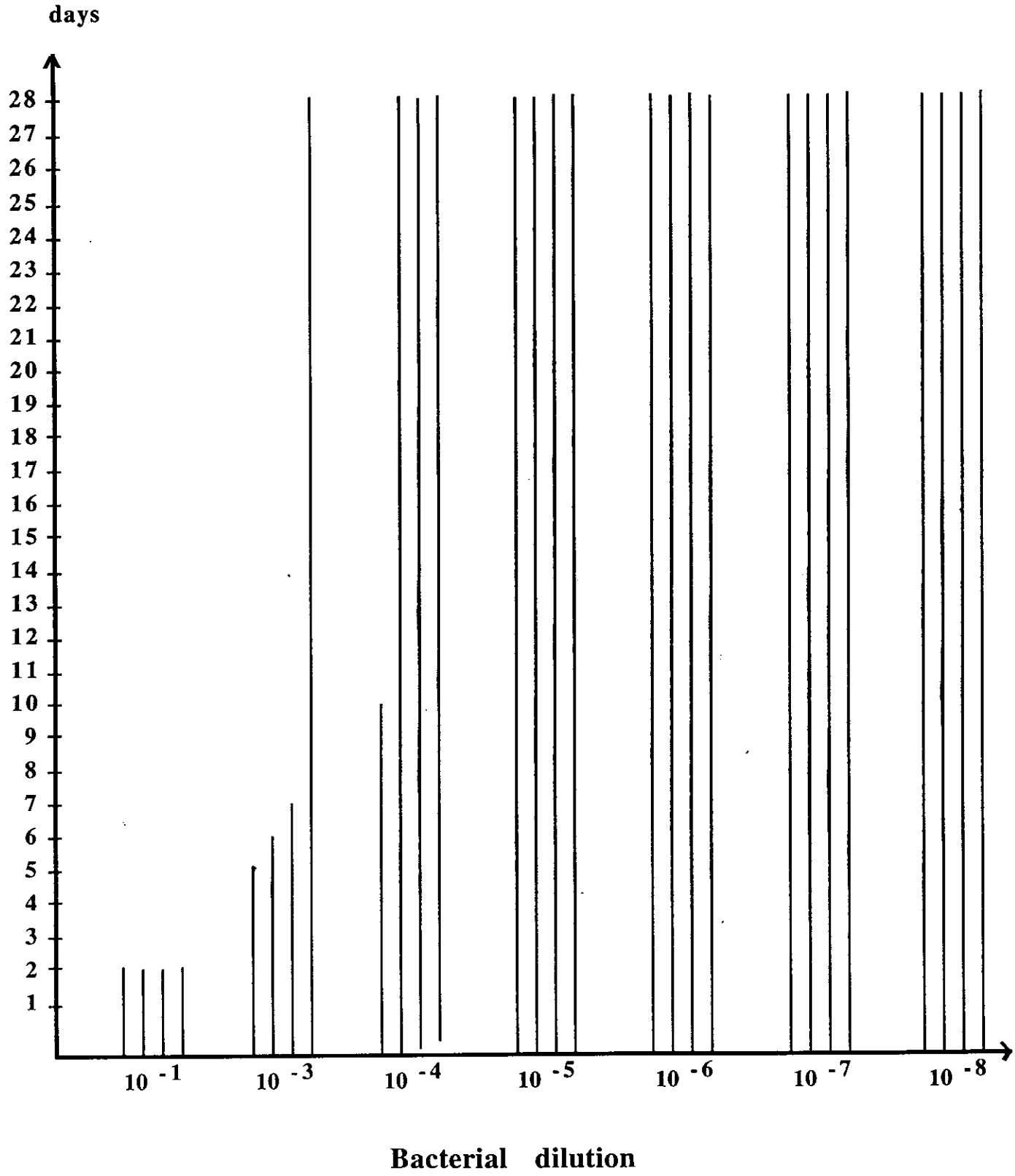
$= -3162$

$= 3 \times 10^{-3}$

$= 3 \times 10^{7}$ C.F.U./ml

(Colony forming Unit/ml).

**Figure (6) : Shows the number of mice Killed by serial dilutions of the mutant form of S. typhimurium strain in each group per day**



# D I S C U S S I O N

Plasmids are defined as extrachromosomal genetic elements capable of autonomus replication. They accur in both Gram-positive and Gram-negative bacteria and may be categorized according to the function they encode (Gottesman, 1981).

The first property of plasmid to be recognised was the ability of the F (fertility) plasmid to transfer chromosomal genes between strains of E. coli which was discovered by tantum and lederberg in 1946 (Willetts and Skurray, 1980).

One of the most common feactures of plasmids is that , they can be eliminated from host cells by various treatments. This process termed "curing" apparently results from inhibition of plasmid replication without parallel inhibition of chromosome replication and as the result of cell division, the plasmid is diluted out (Tomoeda et al., 1983).

Curing may accur spontaneously, but it is greatly increased by treatments that affect DNA replication eg, ultraviolet rays , ionizing radiation , heavy metals as well as growth at elevated temperature (Tomoeda et al., 1983).

Salmonellosis is a continuous problem in man and animals, causing economic losses and public health concern (Woodward et al., 1989) .

Recent studies have indicated that large molecular mass plasmids are required for the virulence of several serotypes of Salmonellae which are capable of producing systemic diseases in man and animals. These include S. dublin S. enteritidis, S. Choleraesuis and S. paratyphi C. (Barrow and Lovell, 1986) .

The exact mechanism by which the virulence plasmids contribute to systemic infection remains to be unclear, it seems that they are important for efficient colonization of spleen, liver and mesenteric lymph nodes after oral

inoculation of mice, but they are not involved in the ability of Salmonellae strains to cross the gut (Montenegro et al., 1991) .

Genetic and physical analysis of these virulence plasmids had led to identifications of genes specifically involved in virulence. A consensus region of 8 kbp, Sal I-XhoI fragment is essential for full expression of virulence. Four virulence genes had been mapped on this region . The different virulence plasmids share large regions of DNA homology as based on DNA hybridization studies (Montenegro et al., 1991) .

Many authors tried to study the presence of plasmid DNA in S. typhimurium strain, depending on examination of different isolates originating from different countries. They studied S. typhimurium strains isolated from Australia, Brazil, Canada, Italy, Romania, Sweden and Yugoslavia (Helmuth et al., 1985 and Williamson et al., 1988).

This work studied the presence of plasmid in S. typhimurium strain which was isolated in Egypt, and tried to isolate and identify this plasmid's size and lastly studied it's role in the virulence of the strain.

For curing of the plasmid, which supposed to be present in S. typhimurium strain, a high temperature of 42°C for 18 hours was used. This method of curing agreed with the work of Paul et al. (1987); where they used high temperature for curing of plasmid in S. gallinarum strains.

Plasmid isolation from the strain was done by alkaline lysis method which is the most commonly used miniprep where plasmid DNA is prepared from small amount of many different cultures (up to 24) of plasmid containing bacteria. This agreed with the work of Barrow and Lovell (1986), where they used the same procedure for isoaltion of plasmid from S. pullorum strains.

Agarose gel electrophoresis was a simple and highly effective method which was used in this study for identification of plasmid DNA which could be visualized by ultraviolet illumination (Ausubel et al., 1987).

Plasmid of S. typhimurium strain was localized in the gel highly above the molecular weight marker (11 kbp), denoted that it is of high molecular weight. This result agreed with Michiels et al. (1987), where they could isolated DNA plasmid of 90 kbp from S. typhimurium strain and that of Gulig and Curtiss (1988), where they had found a plasmid of 100 kbp in S. typhimurium strain, they examined. These scientists used a more complicated method for proper determination of the molecular weight of the plasmid (by restriction enzymes ).

Role of this plasmid in the virulence of the strain was examined by oral inoculation of serial dilutions of both wild (plasmid containing) and mutant (plasmid less) form of the strain, to groups of BALB/C mice, observation for 28 days and determination of $LD_{50}$ of each type of the strain.

$LD_{50}$ of the wild form of S. typhimurium strain was 1 x $10^3$ C.F.U./ml and that of the mutant form of the same strain was 1 x $10^7$ C.F.U./ml. This means that the virulence of the wild strain reduced by $10^4$ fold after curing of the plasmid, which ensure that the plasmid isolated from S. typhimurium strain has a real role in the virulence of the strain.

This results agreed with that of Helmuth and his collegues in (1985), where they found that $LD_{50}$ values obtained for plasmid positive strains of S. typhimurium were up to $10^6$ fold lower than the values obtained for plasmid free strains, where $LD_{50}$ of plasmid positive strains was 1.4 x $10^3$ C.F.U./ml and that of plasmid free strains was 7.0 x $10^8$ C.F.U./ml. this results also agreed with Williamson et al., (1988), which found that cured derivatives of S. typhimurium strains showed reduced virulence by $10^2$ fold following oral infection of mice .