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Molecular diagnosis of salmonella spp.

A study
Submitted to the Council of the College of Veterinary Medicine, University of Al-Qadisiya in Partial Fulfillment of the Requirements for the Degree of Bachelor in Veterinary Medicine & Surgery

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Dedication

To:

My country Iraq …….Peace and safety

The light of my eyes ……my Parents

Hussain ….

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

((فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ ۗ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُلْ رَبِّ زِدْنِي عِلْمًا))

صدق الله العلي العظيم

طه الآية ١١٤

Supervisor certification

I certify that this study , entitled ((**Molecular diagnosis of salmonella spp.**)) , was prepared under my supervision at College of Veterinary medicine/University of Al-Qadisiya in Partial Fulfillment of the Requirements for Bachelor's degree in Veterinary Medicine and Surgery .

Assist. Prof.

Dr. Ahmad Jasim N.

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Certificate of Instructor

We certify that **Hussain abdula jead** has completed the fulfillment of her graduation project entitled **Molecular diagnosis of salmonella spp.** for the year 2016/2017 under our construction.

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By the name of God first who deserves all thanks & pleasure for granting me with well strength & help with which this dissertation has been accomplished peace & prayer on my Bachelor. Guide prophet mohammed & his household .

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Salmonella species are Gram-negative bacilli associated with animal and human infections, which lead to high morbidity rates, not only in the developing world but also in industrialized countries, and the high mortality is mainly observed in the poorest nations of the developing world The epidemiology of *Salmonella*-associated infections varies widely depending on the type of *Salmonella* spp. involved, and despite the improvement in sanitation and hygiene, non-typhoid *Salmonella* (NTS) illnesses continue to impose a significant burden on the population's health in industrialized and underdeveloped countries *Salmonella* infections are transmitted by ingestion of contaminated materials. The most common contaminated foods are ice cream, eggs, shellfish, undercooked meat, raw fruits and vegetables, and contaminated water A major advantage of molecular methods is the reduced time to detection. Faster diagnostic outputs allow earlier epidemiological investigations and infection control interventions Polymerase chain reaction (PCR) is a very powerful technique in molecular biology and is widely used today for an increasing number of applications such as in clinical diagnostics, in identification of individuals DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA

3.1. *Salmonella*

Salmonella species are Gram-negative bacilli associated with animal and human infections, which lead to high morbidity rates, not only in the developing world but also in industrialized countries, and the high mortality is mainly observed in the poorest nations of the developing world. It is believed that epidemics caused by *Salmonella* spp., may have significantly affected the history of human kind, even at present, the effect of *Salmonella* infections on entire communities result in economic burden to developing and also industrialized nations **(Cunha, 2004)**. The interest generated with *Salmonella* spp. as pathogens over the last decades and their implications on history, economics and biomedical science, is represented by close to 70,000 articles posted in Medline, and close to 15 million internet entries **(Sanchez-Vargas *et al*., 2011)**.

3.2. Historical overview

The genus *Salmonella* was ultimately named after Dr. Salmon, an American veterinary pathologist, while Smith was the actual discoverer of the type bacterium (*Salmonellaenterica* serovar choleraesuis) in 1885. Dr. Salmon was the administrator of the USDA (United State Department of Agriculture) research programme, and thus the organism was named after him by Smith **(Cunha, 2004)**. Smith and Salmon had been searching for the cause of common hog cholera and proposed this organism as the causal agent. Later research, however, showed that this organism (now known as *Salmonellaenterica*) rarely causes enteric symptoms in pigs, and was thus not the agent they were seeking (which was eventually shown to be a virus). However, related bacteria in the genus *Salmonella* were eventually shown to cause other important infectious diseases. The genus Salmonella was finally formally adopted in 1900 by J. Lignières for the many species of Salmonella, after Smith's first type-strain *Salmonella* choleraesuis **(Ryan and Ray, 2004)**.

3.3. Morphology

Salmonellae are Gram-negative facultative anaerobic rod-shaped bacteria that measure 0.7-1.5 by 2.0-5.0 μm, non-sporogenic **(Ashton, 1990)**. All are motile with long, peritrichous flagella except two serovars, *Salmonella serovar pullorum* and *gallinarum*. The growth of *Salmonella spp.* is dependent on several factors including temperature, pH, water activity and levels of nutrients present. In general,the optimum temperature to support growth is 37°C, but they can grow over a range of 5 to 43°C and can grow within a pH range of 4.0 to 9.0, with an optimum pH 7.0, the organisms may be protected by organic material such as faeces and litter **(Gast, 1997)**. *Salmonellae* are relatively resistant to environmental conditions and survive well in the presence of moisture. Inactivation occurs quickly on exposure to sunlight and killed by heat treatment **(Shivaprasad, 2003)**. *Salmonella spp.* decline during freezing, though the organism can survive for long time on frozen foods **(ICMSF, 1996)**. *Salmonella* can survive for long periods of time in feed up to 16 months at 25°C and 51% relative humidity **(Williams and Benson, 1987).**The characters *of salmonella* that distinguish it from other enterobacteriacea*Table*(1) *Salmonella* are tolerant of dehydration and salinity, which enable them to persist in the environment **(Acha and Szyfres, 2001).** Most species reduce nitrates to nitrites and they ferment glucose mostly with the formation of gas, urea not hydrolyzed, does not produce indole, lysine and ornithine decarboxylation, and hydrogen sulphide production from thiosulphate on triple sugar iron agar **(D'Aoust, 1989)**. *Salmonella* are oxidase negative, catalase positive, indole and voges-proskauer negative, but methyl red and simmon citrate positive **(Holt *et al*., 2002)**.

Table (1): Biochemical reaction of *Salmonella spp.*

Motility	+	Methyl-Red	+
Capsule	d	Voges-proskauer	-
Glucose (gas)	+	Citrate	+
Lactose (acid)	-	Gelatin	-
ONPG	-	Phenylalanine	-
Sucrose	d	Urease	-
Salicin	d	Hydrogen sulphide	+
Adonitol	-	KCN	-

Dulcitol	D	Gluconate	-
Inositol	-	Malonate	-
Mannitol	+	Lysine decarboxylase	+
Indol	-	Ornithine decarboxylase	+

+ = positive reaction - = negative reaction d =variable reaction (Jawetz *et al*,2001)

Salmonella usually differentiated from other member of Enterobacteriaeceae by some biochemical reaction (Table 2) (Jawetz *et al*.,2001).

Table (2): Differentiating characters of main genera of Enterobacteriaceae.

Genus	Acid from Lactose	Gas from glucose	Motility	Urease	Citrate Utlized	Voges – Proskauer	Growth in KCN
Escheria	+	+	+	-	-	-	-
Shigella	-	-	-	-	-	-	-
Citrobacter	+	+	+	d	+	-	+
Sallmonella	-	+	+	-	+	-	-
Enterobacter	+	+	+	d	+	+	+
Hafnia	-	+	+	-	+	d	+
Klebsiella	+	+	-	+	+	+	+
Serratia	D	d	+	-	+	+	+
Proteus	-	+	+	+	d	-	+
Providencia	-	+	+	-	+	-	+

+ = positive reaction - = negative reaction d= variable reactions (Jawetz et al.,2001)

3.4. Classification and nomenclature

The genus *Salmonella* incorporates Gram-negative, facultative anaerobic rod-shaped bacilli that are classified as members of the family Enterobacteriaceae. This genus, which is estimated to have diverged from *Escherichia coli* approximately 100–150 million years ago, is genetically diverse and has adapted to colonize many different hosts. For example, *Salmonella* bacteria can be found both as commensal and pathogen in a range of warm and cold-blooded animals and they are capable of surviving free in the environment for extended periods of time **(Ryan and Ray, 2004)**. Historically, *Salmonella* naming was based on the original places of isolation such as *Salmonella*

London and *Salmonella* Indiana. However, Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. This was adopted by the International Association of Microbiologists in 1934 **(Scherer and Miller, 2001)**. Agglutination by antibodies specific for the various O antigens is employed to group Salmonella into six serogroups: A, B, C1, C2, D and E. For instance, *S*. Paratyphi A, B, C and *S*. Typhi express O antigens of serogroups A, B, C1 and D, respectively. More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica* subspecies *enterica* **(Andrews and Baumler, 2005)**. Bacteria can also be classified on the basis of phylogeny, and a phylogenetic tree can be derived from the comparison with 16S rRNA or other gene sequences. There are 2463 *Salmonella* serotypes which are now placed under two species due to the difference in 16S rRNA sequence analysis: *Salmonella enterica* (2443 serotypes) and *Salmonella bongori* (20 serotypes). This system is currently used by the WHO collaborating Centre, Centers for Disease Control and Prevention (CDC) and some other organizations **(Pui *et al*., 2011)**. *Salmonella enterica* is further divided into six subspecies, that were categorized by **Tindall *et al*.,(2005)** as follows: *Salmonella enterica* subsp. enterica (subsp. I), *Salmonella enterica* sub sp. salamae (subsp. II), *Salmonella enterica subsp. arizonae* (subsp. IIIa), *Salmonella enterica* subsp. diarizonae (subsp. IIIb), *Salmonella enterica* subsp. houtenae (subsp. IV), and *Salmonella enterica subsp. Indica*(subsp. VI). Subspecies (VII)was described by **Boyd *et al*., (1996**) by multilocus enzyme electrophoresis (MLEE) data. this subspecies is not identifiable by unique biochemical properties. The group originally identified as subsp. Salmonella subsp. bongori (V) is now recognized as the separate species Salmonella bongori **(Reeves *et al*., 1989)**. We represent the *S. Enterica* subspecies with Roman numerals (i.e., I to VII). **(McQuiston *et al*., 2008)**. Before this taxonomy was established, serovar names were wrongly treated as species and hence were italicized. Nowadays, according to the current classification, the familiar names given to serovars, such as *S. enterica serovar Typhimurium*, Enteritidis, or Choleraesuis, are maintained and not replaced by their antigenic formulas. Nonetheless, the nomenclature should be S. enterica followed by the serovar (e.g., *S. enterica serovar Typhimurium*) **(Grimont and Weill,**

2007). *S. bongori* as well as subspecies II, IIIa, IIIb, IV, and VI are rarely isolated from clinical specimens but rather are found principally in cold-blooded vertebrates and in the environment. Therefore, almost all *Salmonella* organisms that cause disease in humans and domestic animals belong to *S. enteric* subspecies enterica (I) **(Grimont and Weill, 2007; and Lan *et al*., 2009)**. *Salmonella* can be classified based on their adaptation to either human or animal hosts: Group 1. *S. typhi*, causes human typhoid fever, and *S. paratyphi* A and B causes paratyphoid fever in humans. Group 2 includes: *S. choleraesuis, S. dublin,* and *S. sendai* which infrequently cause disease in humans but do cause disease in animals.

Group 3 includes: the remaining species, the most important of which are *S. enteritidis* and *S. Typhimurium,* which cause a typhoid-like disease in mice and gastroenteritis in humans **(Acha and Szyfres, 2001; Humphries and Linscott, 2015)**. In spite of the close genetic relationship among serovars assigned to subspecies I, there are significant differences in virulence, host adaptation, and host specificity **(Uzzau *et al*., 2000)**, accordingly, they have been categorized into three different groups: broad-host-range, host-adapted, and host-restricted serovars **(Rychlik*et al*., 2006)**.

1: Serovars Typhimurium and enteritidis are broad-host-range serovars capable of causing systemic disease in a wide range of animals but are usually associated with gastroenteritis in a broad range ofphylogenetically unrelated host species **(Center for Disease Control, 2007)**.

2: Serovars dublin and choleraesuis are host-adapted serovars that are often associated with systemic disease in cattle and pigs, respectively, but can cause disease in other animals, including humans **(Nnalue, 1991)**.

3: Serovars typhi, gallinarum and abortusovis are host restricted serovars that are associated nearly exclusively with systemic disease in human, fowl and ovine **(Pardon *et al*., 1988)**.

3.5. *Salmonella* characterization

Salmonellae are non-fastidious as they can multiply under various environmental conditions outside the living hosts. They do not require sodium chloride for growth, but can grow in the presence of 0.4 to 4%. Most *Salmonella* serovars grow at a temperature range of 5 to 47°C with optimum temperature of35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as54°C **(Gray and Fedorka-Cray, 2002)**. They are sensitive to heat and often killed at temperature of 70°C or above. Salmonella grow in a pH range of 4.0 to 9.0with the optimum between 6.5 and 7.5. They require high water activity (aw) between 0.99 and 0.94 (pure water aw = 1.0) yet can survive at aw < 0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures < 7°C, pH< 3.8 or water activity < 0.94 **(Hanes, 2003)**.

3.6. Epidemiology

The epidemiology of *Salmonella*-associated infections varies widely depending on the type of *Salmonella* spp. involved, and despite the improvement in sanitation and hygiene, non-typhoid *Salmonella* (NTS) illnesses continue to impose a significant burden on the population's health in industrialized and underdeveloped countries **(Westrell *et al.*, 2009)**. It is estimated that 93.8 million cases of gastroenteritis due *Salmonella* spp. occur worldwide leading to 155000 deaths each year, and according to a WHO data from 2001 to 2005, *S. Enteritidis* was the most common serovar worldwide (65% of the isolates), followed by *S. Typhimurium* (12%) and *S.* Newport (4%) **(Majowicz *et al.*, 2010)**. In Africa, *S. Enteritidis* and *S. Typhimurium* represented 26 and 25% of the isolates, respectively, and in Asia, Europe and Latin America, *S.* Entiritidis was the most frequent isolate (38, 87 and 31%, respectively). In North America, *S. Typhimurium* was the most frequently reported (29%) followed by *S. Enteritidis* (21%) and other *Salmonella spp*. (21%) **(Galanis *et al.*, 2006)**. Sub-Saharan Africa hospital-based studies reported blood stream *Salmonella* spp. infections more frequently associated to NTS, particularly *S.* Enteritidis and *S. Typhimurium*, than *S. Typhi* or *S. Paratyphi*. In this region, invasive NTS is endemic and has elevated morbidity

and mortality in children less than 3 years old and adults with human immunodeficiency virus (HIV) infection **(Mandomando *et al*., 2009)**.

In industrialized countries the increasing incidence of NTS has become a public health concern. The estimated NTS-associated illnesses incidence in Europe is 690 per 100000 inhabitants per year. This incidence varies between regions from 240 per 100000 in Western Europe to 2390 per 100000 person years in Central Europe. The incidence of NTS bacteremia in Finland, Australia, Denmark and Canada during 2000-2007 was estimated at 0.81 per 100000 per year **(Laupland *et al*., 2010)**. In the USA, the Food-borne Diseases Active Surveillance Network (FoodNet) found that NTS infections were the most commonly reported (17.6 cases per 100000 inhabitants) and the incidence has not declined since 1996, when the surveillance was initiated, and Food Net data from 1996 to 2005 reported that NTS infections have been the leading cause of death (39%) among food-borne bacterial pathogens with highest mortality among adults more 65 years and highest incidence among children less than 5 years of age (69.5 infections per 100000 children) **(Scallan *et al*., 2015).**The number of multidrug-resistant NTS has increased in many countries since the 1990 report of the multidrug-resistant *S. Typhimurium* DT104 strain that spread around the globe **(Helms *et al*., 2005).** According to the National Antimicrobial Resistance Monitoring System (NARMS), 4.1% of the USA isolates from 2005 to 2006 had decreased susceptibility to cephalosporins and 84% had multidrug-resistance phenotypes, and more comprehensive NARMS data from 1996 to 2007 showed also that invasive NTS were more likely to be multidrug-resistant, but more importantly, it reported that isolates began to show resistance to nalidixic acid (2.7%) and ceftriaxone (2.5%), rising concern about clinical management and public health surveillance and prevention and augmenting the importance of vaccine development **(Crump *et al*., 2011)**.

3.7. Transmission

Salmonella infections are transmitted by ingestion of contaminated materials**(Liu *et al.*, 2015)**. The most common contaminated foods are ice cream, eggs, shellfish, undercooked meat, raw fruits and vegetables, and contaminated water **(Lesser and Miller, 2003)**. Non-typhoid *Salmonella*transmission to humans can occur by consumption of food animal products, non-animal food products, contaminated water, or by contact with animals. Food products mass production and distribution disseminates pathogens rapidly to communities. Furthermore, antibiotic resistance among NTS organisms makes more difficult the control and prevention of these infections **(Majowicz *et al.*, 2010)**. Farm animals are the major reservoir for NTS in industrialized countries with transmission by their contaminated products, and NTS are naturally found in chickens, ducklings, sheep, goats, pigs, reptiles, amphibians, birds, pet rodents, dogs, cats, and in a variety of wild animals making infection control a challenge to public health authorities **(Wacheck *et al.*, 2010)**.

3.8. Pathogenesis of *Salmonella Typhimurium*

The Salmonella spp. are primarily infective for humans (Figure 1), especially (***S. typhi***, ***S. choleraesuis***, and perhaps ***S. Para typhi* A and B**). However, the vast majority of Salmonella are chiefly pathogenic in animals that constitute the reservoir for human infection: poultry, pigs, rodents, cattle, pets and many others**(Jawetz et al,2001)**

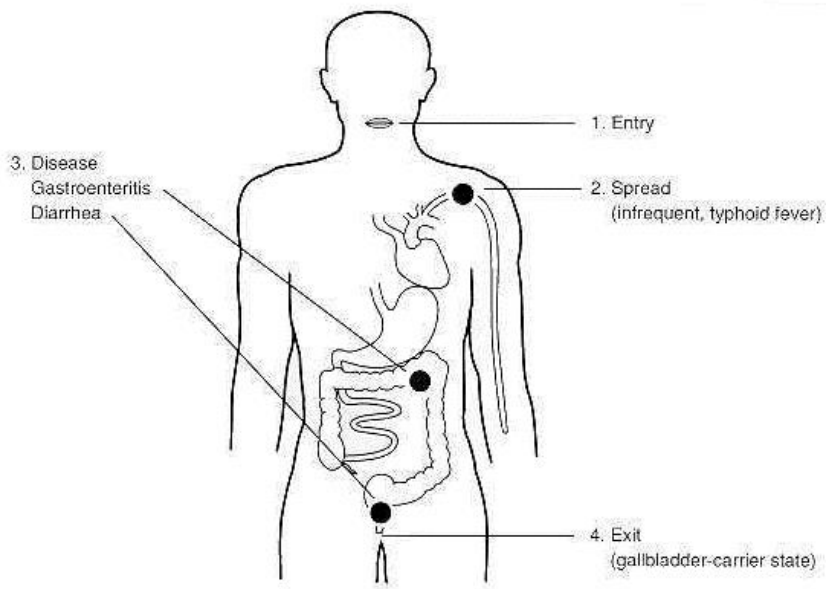

Figure (1): Different sites of infection by *Salmonella spp*.**(Jawetz et al,2001)**

SalmonellaTyphimurium infection begins with the ingestion of organisms in contaminated food or water. The first obstacle to overcome within the host is the acidic pH of the stomach. To protect itself against severe acid shock, *S. Typhimurium* activates the acid tolerance response (ATR), which provides an inducible pH-homeostatic function to maintain the intracellular pH at values higher than those of the extracellular environment **(Foster and Hall., 1991)**. After entering the small bowel, salmonella must reach and traverse the intestinal mucus layer before encountering and adhering to intestinal epithelial cells. In mice, salmonella appear to preferentially adhere to and enter the M cells of the Peyer's patches (PPs) in the intestinal epithelium (Figure 2), although invasion of normally non phagocytic enterocytes can also occur **(Jones *et al.*, 1994)**. Shortly after adhesion, the invasion process appears as a consequence of engaged host cell signaling pathways leading to profound cytoskeletal rearrangements **(Francis *et al.*, 1992)**. These internal modifications disrupt the normal epithelial brush border and induce the subsequent formation of membrane ruffles that engulf adherent bacteria in large vesicles called Salmonella-containing vacuoles (SCVs), the only intracellular compartment in which Salmonella cells survive and replicate **(Francis *et al.*, 1992; Garcia-del Portillo and Finlay., 1994)**. Induction of a secretory

response in the intestinal epithelium initiates recruitment and transmigration of phagocytes from the submucosal space into the intestinal lumen. This process is associated with the production of several proinflammatory cytokines such as tumor necrosis factor alpha (TNF-) and interleukin-8 (IL-8) **(Ohl and Miller., 2001)**. Lastly, the apical epithelial brush border reconstitutes **(Santos *et al*., 2003)**. SCVs are initially integrated within the early endocytic pathway, they need to be later uncoupled to bypass delivery of lysosomal enzymes, This action depends on *Salmonella*-directed changes in host endocytic trafficking and function to eventually avoid fusion with secondary lysosomes **(Garcia-del Portillo and Finlay., 1995 and Rathman *et al*., 1997)**. During SCV maturation, *Salmonella* induces *de novo* formation of an F actin meshwork around bacterial vacuoles, a process which is termed vacuole-associated actin polymerization (VAP) and is important for maintenance of the integrity of the vacuole membrane **(Meresse *et al*., 2001)**. SCVs then migrate to a perinuclear position, in close proximity to the Golgi apparatus, presumably to facilitate interception of endocytic and exocytic transport vesicles to obtain nutrients and/or membrane fragments. This event appears to be essential for bacterial replication **(Deiwick *et al*., 2006)**.

Intracellular *Salmonella* can induce the formation of long filamentous membrane structures called *Salmonella*-induced filaments(SIFs) **(Knodler *et al*., 2003)**. SIFs are tubular aggregates along a scaffold of microtubules and originate from the SCVs and extend throughout the cell. Although the biological role of the induction of SIFs is not completely understood, it has been postulated that this process may lead to an increased availability of nutrients that may otherwise be limited within the SCV **(Rajashekar *et al*., 2008)**.

Salmonella are engulfed by phagocytes. Three types of phagocytes are reported to interact with these invading bacteria: (i) neutrophils, (ii) inflammatory monocytes which differentiate into macrophages, and (iii) dendritic cells, another type of monocytes which functionas antigen-presenting cells, the first two types of cells are both recruited from blood in response to the inflammatory signals **(Cheminay *et al*., 2004; Johansson *et al*., 2006; Geissmann *et al*., 2010)**.

Most of the bacterial cells have breached the epithelium through the M cells, they directly reach the PPs and then the mesenteric lymph nodes (MLNs) via the intestinal lymph, most likely being transported by dendritic cells **(Niess and Reinecker 2006)**. Dendritic cells directly take up bacteria from the intestinal lumen by opening the tight junctions and sending dendrites to the lumen **(Rescigno *et al.*, 2001)**. Inflammatory monocytes (macrophages) are those phagocytes which accumulate predominantly in PPs and MLNs, followed by neutrophils **(Rydstrom and Wick., 2007)**. Salmonella are then phagocytosed and internalized again within SCVs, triggering a response similar to that reported inside epithelial and M cells to ensure bacterial survival and replication **(Ohl and Miller 2001; Rydstrom and Wick 2007)**. Migration of these infected phagocytes, predominantly macrophages, facilitates systemic dissemination of the bacteria via the bloodstream to several additional tissues, such as the spleen and liver, where this pathogen preferentially replicates **(Ohl and Miller, 2001)**.

Alternatively, direct blood access of *Salmonella*-infected phagocytes from the basolateral side of the intestine has also been suggested to contribute to systemic dissemination. This hypothesis is supported by the finding of infected phagocytes in the blood within minutes after oral infection and is attributed to a manipulation of the motility of the infected cells **(Worley *et al.*, 2006)**.

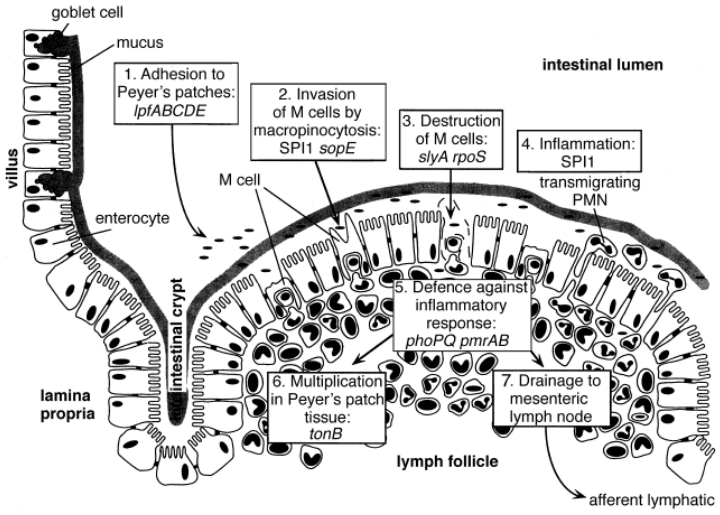

Figure (2): Colonization of murine Peyer's patches by *S. Typhimurium*. Cross-section of the intestinal wall at the area of a Peyer's patch showing a lymph follicle (centre) and part of an intestinal villus (left). Consecutive stages of Peyer's path colonization and *S. enterica* virulence required during infection.

3.9. Virulence factors and strategies

S. Typhimurium possesses many virulence strategies employed tointeract with the host defense mechanisms. The majority of the genes encoding the most important virulence factors are located within highly conserved *Salmonella* pathogenicity islands (SPIs), whereas others are found on a virulence plasmid (pSLT) or in the chromosome **(Fàbrega and Vila., 2013)**. Together with further virulence components such as the pSLT plasmid-carried *spv* operon, several types of adhesins, flagella, and the essential components for biofilm formation **(Coburn *et al*., 2007)**.

3.10. Pathogenicity islands

Pathogenicity islands (PIs) are genetic elements that carry genes encoding virulence factors, such as adhesion, invasion, and toxin genes **(Hacker *et al*., 1997)**. Pathogenicity islands were first identified in uropathogenic *E. coli* (UPEC) in the late 1980s, and have since been described in a wide variety of bacteria **(Hacker, *et al*., 1997)**. Pathogenicity islands have been identified in both Gram- negative and Gram-positive species, and are associated with plant, animal, and human pathogens, as well as non-pathogenic bacteria. They typically harbour large clusters of genes (10 – 200 kb) related to virulence and/or survival and fitness, and have a different GC content in comparison to the rest of the genome. Pathogenicity islands can often be mosaic in structure and are often bordered by transposon insertion sequences and direct repeats, as well as bacteriophage genes, indicating that their insertion into the genome occurred via single or multiple horizontal gene transfer events **(Hacker & Kaper., 2000)**. Clusters of chromosomal virulence genes, termed *Salmonella* pathogenicity islands (SPIs) provide a molecularbasis for understanding *Salmonella* pathogenicity **(Amavist *et al*., 2003)**.

To date there have been 21 Salmonella pathogenicity islands (SPIs) identified.**(Haneda *et al*., 2009; Sabbagh *et al*., 2010)**.

3.11. Other potential virulence factors

A-Fimbriae

Fimbriae or pili are proteinous structures found on bacteria that can mediate interaction with cells, fimbriae are normally specific to a receptor and can be used at different critical times during the infection, each serovar harbours a unique combination of fimbrial operons **(Sabbagh *et al*., 2010)**. Whole genome sequence analysis revealed eight putative fimbrial operons shared by both *S. Typhimurium* and S. Typhi [bcf, csg(agf),fim, saf, stb, stc, std, sth] **(McClelland**

***et al*., 2001; Parkhill *et al*., 2001)**. Salmonella enterica serovar Typhimurium possesses five unique fimbrial sequences known as lpf, stf, pef, sti and stj **(McClelland *et al*., 2001)**. These unique fimbriae were not involved in systemic colonization of the spleen, and Lpf was shown to be involved in intestinal colonization of mice **(Weening *et al*., 2005)**. A type IVB pilus located on SPI-7 is only found within the *S. Typhi* genome, along with five other unique fimbrial operons (sef, sta, ste, stg an dtcf) **(Parkhill *et al*., 2001)**. For the majority of these fimbriae, little is known about their true function, expression conditions or their importance for virulence during infection. Type IV pili are used by Typhi for adhesion to human monocytes and epithelial cells by interaction with the cystic fibrosis transmembrane conductance regulator receptor **(Tsui *et al*., 2003; Pan *et al*., 2005)**. Tcf was recognized by human sera from patients with typhoid **(Harris *et al*., 2006)** and Stg mediates adherence to epithelial cells and reduces phagocytosis **(Forest *et al*., 2007)**. All fimbrial operons are intact in *S.Typhimurium* strain LT2, although pseudogenes are found within six fimbrial operons of*S.Typhi* strain CT18 (fimI, safE, sefA, sefD, bcfC, steA, stgC, sthC, sthE) **(Townsend *et al*., 2001)**. The unique repertoire of fimbrial adhesion systems may explain in part some differences observed between the host tropism colonization niches of these two serovars.**(Sabbagh *et al*., 2010)**.

B-Flagella

In Salmonella, the major subunit of flagella is usually encoded by fliC or fljB, which correspond to the H1 and H2 variants of the H antigen, respectively (Silverman & Simon, 1980). Only one type of flagellin can be expressed at a specific time by a mechanism known as phase variation **(Simon *et al*., 1980)**. This antigenic variation can be observed in *S. Typhimurium*, but most *S.Typhi* strains are considered monophasic, as they lack a corresponding fljB locus **(Frankel *et al*., 1989)**. However, some S. Typhi isolates from Indonesia contain a linear plasmid encoding a novel flagellin, fljBz66, but reversion to fliC is considered irreversible due to a deletion **(Baker *et al*.,**

2007).fliB, involved in methylation of the flagellin in *S. Typhimurium*, is a pseudogene in *S. Typhi***(Parkhill *et al.*, 2001)**.

C-Vi antigen

The Vi antigen is a polysaccharidic capsule absent in *S.Typhimurium* and present in S. Typhi. Vi is important for virulence and is controlled by two loci: viaA and viaB **(Kolyva *et al.*, 1992)**. The viaB locus located on SPI-7 is composed of two operons: tvi ABCDE and vex ABCDE. The Vi capsule causes several differences between *S. Typhimurium* and *S. Typhi* at the level of the host's response to infection. The Vi capsule is associated with inhibition of complement activation, resistance to serum and to phagocytosis and is involved in survival inside phagocytes **(Achouri*et al.*, 2015; Miyake *et al.*, 1998)**. The via B locus lowers the invasiveness of the bacteria towards epithelial cells, as via B mutants are super invasive **(Zhao *et al.*, 2001)**, and *S.Typhimurium* harbouring the viaB locus is less invasive **(Haneda *et al.*, 2009)**. Tvi A avoids interleukin-8 production in the intestinal mucosa by repressing flagellin secretion, which reduces the recognition and activation of Toll-like receptor (TLR)-5 **(Raffatellu *et al.*, 2005; Winter *et al.*, 2008)**. Vi also prevents the recognition of lipopolysaccharide by TLR-4 and reduces inflammation in the intestinal mucosa **(Sharma and Qadri., 2004; Wilson *et al.*, 2008)**. *S.Typhimurium* sets off an immune response, which causes inflammation characterized by an important neutrophil influx that may be the result of its lack of capsule. Thus, Vi allows S. Typhi to disseminate systemically in its human host by crossing intestinal cells without activating the immune response, promotes resistance to killing by serum and contributes to survival inside phagocytes **(Raffatellu *et al.*, 2006)**. Vi is a protective antigen and the actual constituent of the parenteral typhoid fever vaccine. **(Sabbagh *et al.*, 2010; Waddington*et al.*, 2014)**.

3.12. Salmonella Cell wall

The discovering of electronic microscope play an important role in the development of bacterial cell wall studying, the cell wall is the principal stress –bearing and shape –maintaining element in bacteria and its integrity was of critical importance to cell viability **(Gitai, 2005).**

The first scientific researcher Weidal and his team in 1960 whom studied and described the cell wall of Gram-negative bacteria (*E.coli* as a model).Its a complex compound was consistingof three layers (Figure 3) that is inner into outer side as follow:

1. Inner (cytoplasmic) membrane
2. Peptidoglycan Layer
3. Outer membrane layer, also called L-Layer because it has lipopolysaccharide which lying on the outer surface of bacterial cell **(Caldwell *et al*., 1981).**

3.12.1. Outer membrane Layer

Most structural and functional studies were conducted on outer membrane of cell wall of *S. enterica* by **(Makela and Mayer, 1976)**.They showed its highly specific structure outside of peptidoglycan and plasma layers and it intermediate line between the cell and their environment.

It is a true membrane because it contains a phospholipid and proteins inpercentage similar to that biological membranes.Their specialized function depend on their compositions which are included:-

1. Lipopolysaccharide
2. Many major proteins with high specifity and several of minor or secondary proteins which are composed the matrix **(Huang *et al*., 2006).**

The outer membrane represent a number of important roles for the cell, A) it represents a barrier to the entry and exit of both beneficial and harmful molecules,B) the outer membrane function to control C) the movement of molecules, they facilitate the uptake of nutrients and

excretion of toxic molecules and also D) prevents the entry of deleterious molecule **(Nikaido, 2003).**

Outer membrane proteins also represent attachment sites for bacteriophage. In addition, outer membrane proteins and structures such as flagella and LPS are the features recognized by the immune system of eukaryotic cells **(Postle and Kadner, 2003).**

3.12.2. Lipopolysaccharide

Lipopolysaccharide is the major part of the outer membrane of the cell wall of Gram-negative bacteria ;it is a potentially toxic natural compound found inside Gram-negative bacteria. In the 1800 century it became understood that bacteria could secret toxins into their environment.While term endotoxin came from Richard Pfiffer who discovered that in 1892, in his work with *Vibrio cholera* **(Schachter, 1999).**The comparison between classic exotoxin and endotoxin of bacteria may be explained in Table(3).

Table (3): Characteristics of bacterial endotoxins and classic exotoxins (Schachter, 1999).

Proerty	Endotoxin	Exotoxin
Chemical nature	Lipopolysaccharide(Mw=10 KDa)	Protein (Mw=50-1000KDa)
Relationship to cell	Part of outer membrane	Extracellular
Denatured by boiling	No	Usually
Antigenic	Yes	Yes
Form toxoid	No	Yes
Potency	Relatively Low(> 100 mcg)	Relatively high (1ml)
Specificity	Low degree	High degree
Enzymatic activity	No	Usually
Pyrogencity	Yes	Occasionally

Lohmann and Barton (2004)clarified that endotoxin and LPS are interchangeable term and defined lipopolysaccharide as purified bacterial extracts which are reasonably free of detectable contaminations, particularly protein .Such preparations may be obtained by using the Westphal extraction procedure as a starting point.

In contrast, the term endotoxin should be used to refer to products of extraction procedure which result in macromolecular complex of LPS, protein and phospholipid complexes.These complexes are normally obtained by extraction of bacteria with trichloroacetic acid, EDTA or butanol. The purified LPS described by **Rietschel *et al.*, (1996)** as complex glycolipid composed of biological active lipid (Lipid -A) which linked to a polysaccharide.

3.12.2.1 Lipopolysaccharide (LPS) structure

Lipopolysaccharide (LPS) is an integral part of the outer membrane, and by weight LPS makes up about 10 % of cell wall of Gram –negative bacteria .A single cell contains approximately 3.5×10^6 LPS molecules, that makes up about 75%of outer layer of cell wall (Figure 3). **(Marshall *et al*., 2004).**

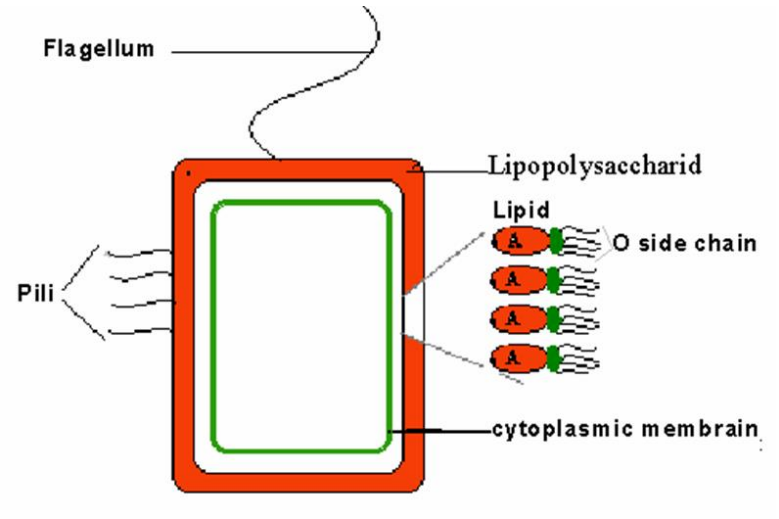

Figure (3): The cell wall of Grame-negative bacteria. **(Lohmann and Barton ,2004).**

Lipopolysaccharides are complex amphilic molecules with a molecular weight of about 10 KDa that vary widely in chemical composition both between and among bacterial species. The architecture of lipopolysaccharide of most species of Enterobacteriaecae (Figure 3) is built up of three separate building blocks: **Erridge *et al*.,(2000).**

1. **Lipid A:** Is the lipid component of LPS; it contains the hydrophobic membrane –anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6-7saturated fatty acids attached.Some fatty acids are attached directly to the (NAG) dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present **(Schromm *et al*., 2000).**
2. **Core region (R) polysaccharide:** The core region consists of an inner and an outer core.

A- Inner core: it consistsof two or more 2-keto-3-deoxyoctonic acid (KDO) sugars linked to the lipid A and two or three heptose (L-glycero-D-manno-heptose) sugars linked to the KDO. The smallest LPS molecule produced by Gram- negative bacteria under natural conditions is Re -LPS (Lipid A with one or two KDO sugars), but longer LPS molecules are more common **(Currie and Poxton, 1999)**.

B- Outer core

It consists of common sugars and is more variable than the inner core. It is normally three sugars long with one or more covalently bound sugars as side chains. LPS serotypes consisting of lipid A and complete inner and outer core are denoted Ra-LPS, whereas the Rb-and Rc-LPS serotypes only contain a part of the outer core **(Amor *et al*., 2000).**

3. The O antigen or O side chain

This part of LPS molecule is attached to the terminal sugar of the outer core, extends from bacterial surface(Figure 4), it consists of repeating oligosaccharide subunits made up of 3-5sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide and it maintains the hydrophilic domain of the LPS molecule. Each unit is composed of three sugars with a single sugar connected to the first and third sugar of the unit **(Raetz, 1990).** O side chain is a major antigenic determinant (antibody–combining site) of the *Salmonella spp* cell wall,and the biochemical analysis of this chain has established a regular sequence of sugars, these repeating sequences may beeither linear from one side or more side branches of the component sugars (Figure 5&6) **(van Amersfoort *et al*., 2003),** The length and the composition of these repeating units vary from strain to strain **(Glauser *et.al*., 1991).**

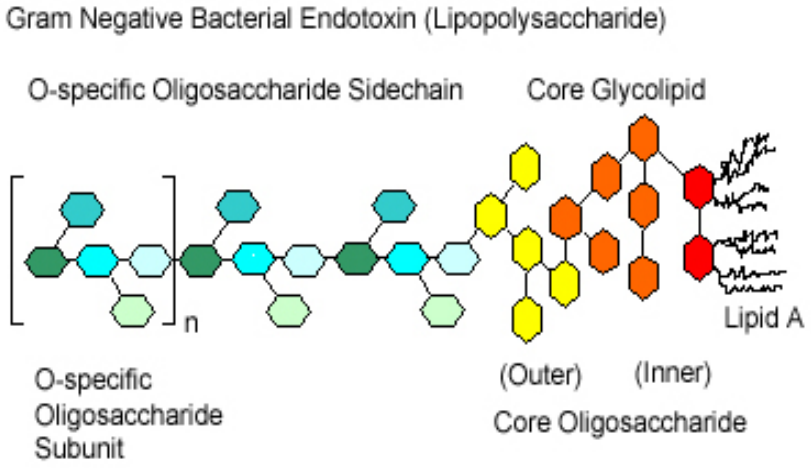

Figure (4): Architecture of LPS of Gram- negative bacteria **(Erridge *et al*., 2000).**

Basic Structure	O-polysaccharide O-polysaccharide O-polysaccharide	R-Core	Lipid Moiety
Function	Antigenic Specificity	Link between polysaccharide and lipid moieties	Biological Activity (Toxicity)
Chemical Composition	1) Hexoses 2) Hexosamines 3) Deoxyhexosamines 4) 6-Deoxyhexoses 5) 3,6-Dideoxyhexoses	1) Hexoses 2) Hexosamine 3) Heptose 4) Octose (acid) 5) O-phosphorylethanolamine 6) Phosphate	1) Fatty acids (C_{10} to C_{22}) 2) Glucosamine 3) Ethanolamine 4) Phosphate
Specific Chemical Components	1) D-Galactose D-Glucose 2) D-Galactosamine D-Glucosamine 3) L and D-Fucosamine D-Viosamine 4) L-Fucose L-Rhamnose 5) Abequose Colitose Paratose	1) Glucose Galactose 2) N-acetylglucosamine 3) L-glycero-D-mannoheptose 4) 2-Keto-3-deoxyoctonate (KDO)	1) 3-hydroxymyristic acid Myristic acid Lauric acid Palmitic acid

Figure (5): Function and chemical composition of lipopolysaccharid molecule.Note: The numbers of the specific chemical components are correlated with the numbers in the chemical composition above. C=carbon **adopted from Lohmann and Barton, (2004)**

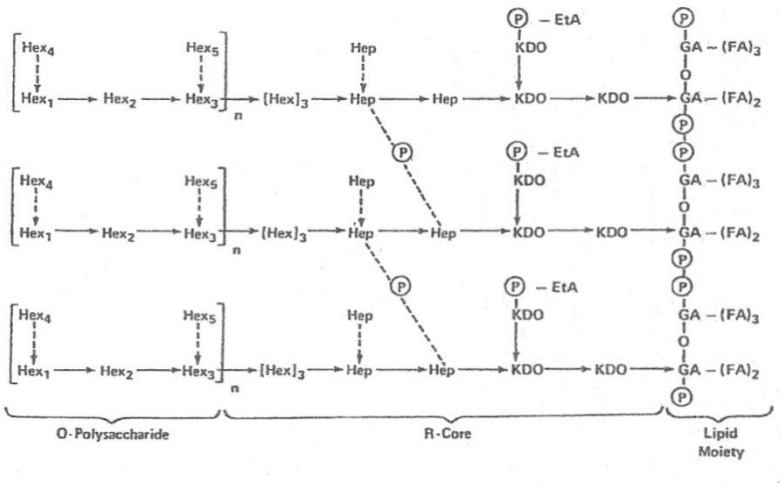

Figure (6): The general structure of lipopolysaccharid molecule.Hex=hexoses; Hep=heptoses; P=phosphate; KDO=2-keto-3-deoxyoctonate; EtA=ethanolamine; GA=glugosamine ; FA=fatty acid **(adopted from Lohmann and Barton, 2004)**

LPS molecule with O antigen are denoted S-LPS. Colonies from bacteria with O-antigen –containing LPS have a smooth (S) appearance on the plate, while bacteria that express an O –antigen –lacking LPS have a rough (R) appearance **(Rietschel *et al*., 1994)**.The rough LPS molecules are denoted further as Ra, Rb, ect to indicate the length of core region .In Re –LPS which is called `deep rough LPS` the core region is reduced to a 2-keto-3-deoxyoctonate (KDO), so that Re –mutants are often used to rise antibodies against the core region in attempt to provide cross –protection against variety of bacterial species **(Lohmann and Barton, 2004)**. Then, the core region in the middle portion of the LPS molecule, which contains inner and outer parts (Figure 2).The chemical analysis of the R-core has shown five basal sugars, phosphate and O-phosphorylethanolamine (Figure 3), the inner core link the lipid moiety to the O-specific side chain by attachment of 2-keto-3-deoxyoctonate (Figure 4), so the synthesis of minimal core is essential for the survival of bacteria and the smallest naturally occurring LPS structure consists of lipid A and KDO **(Mackay,2002)**.

The lipid- A (glycophospholipid): which refers to all chemical compounds of LPS that become insoluble after the acid hydrolysis. Lipid A is a composed entirely of carboxylic acid (fatty acids = C10 - C22).There are ten different carboxylic acids are found in a mutant of *S.Typhimurium*

(Figures 3 & 4). Variation in the lipid-A structure was found between Gram- negative bacteria that will affect the number, length and position of fatty acids, also the backbone structure; In addition to that Lipid-A mediated most of the toxic effects of endotoxin **(Ulevitch and Tobias, 1995)**. According to the Figure (4) phosphate group in the LPS molecule were known to be essential for the biological activity of this bacterial product, and the studies showed that the toxicity of endotoxin was reduced after exposure to alkaline Phosphatase preparation **(Poelstra *et al*., 1997)**.

3.12.2.2 Biological activity of Lipopolysaccharide

Lipopolysaccharide are toxic to most mammals, lethal dose for human 1 ng/1 ml plasma, and LPS causes death 30-80 % in septic shock. LPS produce the same range of biological effects in animal host, and the toxicity of LPS is associated with lipid A and immunogenicity is associated with polysaccharide component. The injection of living or killed Gram- negative bacteria or purified LPS into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as: fever, change in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death. **(Erridge *et al*., 2000)**. The physiological effects of LPS (endotoxin) are thought to be mediated by lipid A, Since lipid A is embedded in the outer membrane of bacterial cells, it exerts its toxic effects when related from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the membrane attack complex (MAC). Ingestion and killing by phagocytes, or killing with certain types of antibiotics. **(Katharina and Barton, 2004)**. It is thought that LPS released into the blood stream by lysing Gram-negative bacteria is first bound by certain plasma proteins identified as LPS –binding protein. The LPS-binding protein complex interacts with CD_{14} receptors on monocytes and other types of receptors on endothelial cells **(Cohen, 2000)**.In monocytes (macrophages) three types of events are triggered during their interaction with LPS:

A-Production of cytokines

Including 1L-1,1L-6,1L-8,tumor necrosis factor (TNF)and platelet activating factor (PAF),this in turn stimulate production of prostaglandin and leukotrienes .These are powerful mediators of inflammation and septic shock that accompanies endotoxemia. LPS activates macrophages to enhanced phagocytosis and cytotoxicity, and macrophages are stimulated to produce and release lysosomal enzymes, TNF, IL-1 (endogenous pyrogen), as well as other cytokines and mediators **(Blunck *et al*., 2001)**.

B-Activation of the complement cascade

C_3a and C_5a cause histamine release (leading to vasodilation)and effect on neutrophil chemotaxis and accumulation and the result is inflammation **(Sehic *et al*., 1998)**.

C- Activation of the coagulation cascade

Initial activation of Hageman factor (blood-clotting factor XII), can activate several humoral system resulting:

1. Coagulation: A blood clotting cascade that leads to coagulation, thrombosis, acute disseminated intravascular coagulation, which depletes platelets and various clotting factor resulting in internal bleeding.
2. Activation of the complement alterative pathway which lead to inflammation.
3. Plasmin activation which lead to fibrinolysis and hemorrhaging **(Dekkers *et al*., 2000; Pawlinski *et al*., 2003).** The net effect is to induce inflammation, intravascular coagulation, haemorrhage and shock .

LPS also act as a B –cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM **(Rietschel *et al*.,1994).**

3.12.2.3. Extraction of Lipopolysaccharide

The first isolation of LPS was in 1935 by **Boivin and Messrobeanu** via using trichloroacetic acid at 4 C, while **Ribi** and his team were used ether at (6-12)C and **Robert** was used water in LPS extraction at 80 °C and finally **Westphal and Jann, (1965)** were used hot phenol –water as the most common procedure for LPS extraction in 1952.

As general there are at least (18) lipopolysaccharide extraction methods were available, but the Westphal method with some modifications are the perfected method that applied by many researchers **(Ridley *et al*., 2000)**.

The products of LPS extraction method may be purified either by washing with distal water many times accompanied with centrifuged with high speed (100,000 rpm/1 hrs), or purified by pass the products on sephadex G –200 gel by used column (2.5×96 cm) or by using sepharose 4 B or sepharose 6 B by used column (2.5× 40 cm) **(Galanos and Luderitz, 1993)**.

3.13. Outer membrane proteins

Approximately 50% of the dry mass of the outer membrane of Gram-negative bacteria consists of proteins and more than 20 immuno-chemically distinct proteins (termed outer membrane proteins OMPs) have been identified in Gram-negative bacteria.

These proteins were represent the major structure of outer membrane in Enterobateriaecae with molecular weight which varaint depending on species **(Dutzler *et al*., 1999).**

3.13.1.Outer membrane proteins were classified into two groups

1. **Major outer membrane proteins**

Some of the major outer membrane proteins of a number of Gram-negative organisms have been shown to transverse the outer membrane and to function as porins, permitting the flow of small molecular weight compounds from the external milieu to the periplasmic space and vice

versa **(Dirienzo *et al*., 1998)**. These proteins also classified into three groups according to their functional properties.

2. **Porins**

Porins are a family of hydrophobic proteins located in the outer membrane of the cell wall of Gram-negative bacteria. Porin is a trimeric membrane proteins and their function as a diffusion pore with a dimaeter of about 1 nm in outer membrane of *Salmonella*. These porins are permeable to molecules with molecular masses lower than approximately 600 dalton **(Henderson *et al*., 1996).**

There are several porins in *Salmonella* Typhimurium:

1. Matrix porins (OmpF), 1.2 nm diameter of pores.
2. Osmoporins (Ompc and Ompd), 1.1 nm diameter of pores and these pores are facilated cations permasses.
3. Phosphate starvation –induce porin (pho E).

These porins was appeared as three bands on gel of SDS-PAGE electrophoresis with molecular weight approximately 35-38 Kda **(Hernandez *et al*., 1999)**. **Nikaido, (2003),** was reported the important role of these porins in the virulence of *entrobacteracea* by their capacity to induce synthesize of many proinflammatory cytokines and their stability against proteolysis reaction.

4. Heat modifiable protein (Omp A)

Omp A is one of abundant proteins of outer membrane of common Gram- negative bacteria espacially *enterobacteracea*as*Salmonella Typhimurium* and *E coli* which serves as cell surface receptors for certain bacteriophage and colicins. Omp A protein is important for maintained of the outer membrane integrity .Also its play a role inability of bacteria to act as recipients in the conjugation with F^+ donors and it contributed in pass un chargeable high molecular weight mass through ionic channels that ompA protein was conformed **(Power *et al*., 2006)**.

5. Lipopotein

Lipoproteins are found in bacterial cytoplasmic membrane and are also common constituents of cell wall of Gram-negative bacteria, and they are the most abundant outer membrane protein of*Salmonella yphimurium* and *E.coli* which is responsible for anchoring the outermembrane to the peptidoglycan layer.These lipoproteins are hydrophilic with molecular weight about (5-9) KDa and have α-helical structure **(Hellman *et al*., 2000).**

6. Minor outer membrane proteins

These proteins are less than major OMPs and it in similar to porins in some character, like Lam B protein which act as receptor for lambda bacteriophage and it responsible for passive diffusion of maltose and maltodextrin molecules through outer membrane, whereas TSx protein act as T6 bacteriophage receptor and responsible for nucleosides diffusions. Other minor OMPs like Ton A, Cir and Blub, and their molecular weight approximately about 26-83 KDa **(Brooks *et al.*, 2001)**.

3.14. Serotyping

Serotyping is a phenotypic technique used for characterization of *Salmonella* isolates that is based on immunological reactivity of specific antibody against various combinations of the surface thermostable O somatic (cell wall) and thermolabile H(flagella) antigens (**Grimont andWeill., 2007**). The antigenic formulae of serotypes are defined by the WHO collaborating center for Reference and Research on *Salmonella* at the Pasteur Institute, France, and the new serotypes are listed in the annual updates of the Kauffman-White scheme (**Grimont and Weill.,2007**). Serotyping is essential tool that can be used for investigation of *Salmonella* outbreaks (**Clark *et al.*, 2009**). The commercially available and commonly used slide agglutination test is based on specific O and H anti- Salmonella sera, the antiserum is either is a mixture of mono or poly-clonal antibodies produced invitro or invivo in different animal species including the mouse and rabbit, in principle, the serum and the isolate are mixed on a glass slide and a visible agglutination representing an antibody- antigen complex will be formed if matching antigens are present (**Quinn *et al.*, 1994**). The total number of *Salmonella* serotypes is 2579, including 2557 and 22 for *S. enterica* and *S. bongori* respectively (**Grimont and Weill, 2007**). *S. enterica* strains can also be classified on the basis of the O (lipopoly saccharide) surface antigen into 67 serogroups when strains are differentiated by both their O and H (flagellar) antigens. **(Fàbrega and Vila., 2013**).

3.15. Serological tests

Include the macroscopic tube agglutination test, rapid serum test, stained antigen whole blood test and micro agglutination test using tetrazolium-stained antigens **(Shivaprasad, 2000)**. Also Enzyme-linked immunosorbent assays (ELISA) which conceder rapid and have high sensitivity and specificity relative to microbiological culture. These are especially useful for large scale screening of poultry flocks **(Barrow, 2000)**. However, results may be difficult to interpret where birds have been vaccinated. All tests are subject to false positive reactions, and all positive serological tests should be confirmed by culture of samples from the suspect bird **(Waltman *et al.*, 1998)**. Commercially-available kits and research-type protocols exist for measuring anti-*Salmonella* immunoglobulin in serum, with commercial ELISA assays measuring IgG on flagellin-coated plates and research ELISA assays capable of measuring IgA or IgG on LPS or flagellin coated plates **(Holt and Porter, 1993)**. Another traditional subtyping technique that is also based on surface–associated antigens that are detected by antibodies directly labeled with fluorescein or a fluorescien–labeled conjugate which is added for the visualization of the antigen – antibody binding **(Towner and Cockayene, 1993)**.

3.16. Molecular Diagnosis

A major advantage of molecular methods is the reduced time to detection. Faster diagnostic outputs allow earlier epidemiological investigations and infection control interventions **(Schuurman *et al.*, 2007)**. Polymerase chain reaction (PCR) is a very powerful technique in molecular biology and is widely used today for an increasing number of applications such as in clinical diagnostics, in identification of individuals. The polymerase chain reaction (PCR) is an *in vitro* technique used to replicate, or amplify, a specific region of DNA billions-fold in just a few hours **(Saiki *et al.*, 1988; Mullis and Faloona, 1987)**. PCR has been adopted for rapid detection of *Salmonella* in food, in the

environment **(Soumet *et al.*, 1999)** and in clinical specimens **(Schrank *et al.*, 2001)**. There are, a lot of attention have been focused on PCR techniques to detect *Salmonella*. PCR is used in diagnostic and research laboratories to generate sufficient quantities of DNA to be adequately tested, analyzed, or manipulated. PCR has rapidly become a standard method in diagnostic microbiology **(Loeffelholz and Deng, 2006)**. Multiplex PCR assay would offer an effective alternative to traditional typing methods for the identification and differentiation of the most clinically relevant *Salmonella* types **(Alvarez *et al.*, 2004 and Yukawa *et al.*,2015).** Multiplex PCR has become an important tool of modern molecular biology because it offers several important advantages over amplifications involving one DNA marker at a time or "monoplex". First, the cost of analysis and labor to obtain a set of results from multiple markers can be reduced. Second, the amount of information obtained per unit time of investment improves. Third, the amount of template DNA required to obtain results is reduced **(Chamberlain *et al.*, 1988)**. Multiplexed reactions are carefully balanced by optimizing the reaction conditions and primer sequences so that one locus with its respective primer set does not preferentially amplify over the others **(Butler *et al.*, 2001)**.

3.16.1. DNA sequencing

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology, forensic biology, and biological systematics. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species **(Petterssone*t al* ., 2009)**. DNA sequencing were made during

the 1970s. Frederick Sanger developed rapid DNA sequencing methods at the MRC Centre, Cambridge, UK and published a method for "DNA sequencing with chain-terminating inhibitors" in 1977 **(Sanger *et al*., 1977)**, while Walter Gilbert and Allan Maxam at Harvard also developed sequencing methods, including one for "DNA sequencing by chemical degradation" **(Maxam &Gilbert1977; Gilbert, 1981)**. Allan Maxam and Walter Gilbert developed a method for sequencing single-stranded DNA by taking advantage of a two-step catalytic process involving piperidine and two chemicals that selectively attack purines and pyrimidines. Purines will react with dimethyl sulfate and pyrimidines will react with hydrazine in such a way as to break the glycoside bond between the ribose sugar and the base displacing the base (Step 1). Piperidine will then catalyze phosphodiester bond cleavage where the base has been displaced (Step 2) **(Maxam & Gilbert, 1977)**, while Frederik Sanger was developing an alternative method. Rather than using chemical cleavage reactions, Sanger opted for a method involving a third form of the ribose sugars. Sanger proceeded to establish a protocol in which four separate reactions **(Sanger *et al*., 1977)**. The advent of Sanger sequencing gave a boost to DNA sequencing in general and led to an even more rapid accumulation of sequence data for various genes and organisms. This increase in sequence data in the scientific literature also resulted in the establishment of the first DNA sequence repository by Walter Goad at Los Alamos National Laboratories in 1979. This repository has since become GenBank **(Mount, 2001)**. DNA sequencing reactions can be carried out in a single reaction tube and be prepared for loading once the reaction reagents had been filtered out. The capillary system is set up to deliver new polymer to the capillary, load the sequencing reaction into the capillary, apply a constant electrical current through the capillary, and have the resolved fragments migrate past an optical window where a laser would excite the dye terminator, a detector would collect the fluorescence emission wavelengths, and software would interpret the emission wavelengths as nucleotides (Figure 7). Such systems can deliver 500−1000 bases of high quality DNA sequence in a matter of a few hours **(Zhang *et al*., 1997)**.

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استنتاج

أنواع السالمونيلا هي عصيات سلبية الجرام ترتبط بالعدوى الحيوانية والإنسانية، مما يؤدي إلى ارتفاع معدلات المراضة، ليس فقط في العالم النامي ولكن أيضا في البلدان الصناعية، ويلاحظ ارتفاع معدل الوفيات بشكل رئيسي في أفقر دول العالم النامي علم الأوبئة من الالتهابات المرتبطة بالسالمونيلا تختلف اعتمادا كبيرا على نوع السالمونيلا النيابة. وعلى الرغم من التحسن في الصرف الصحي والنظافة الصحية، فإن الأمراض غير التيفية السالمونيلا (نتس) لا تزال تفرض عبئا كبيرا على صحة السكان في البلدان الصناعية والمتخلفة تنتقل العدوى بالسالمونيلا عن طريق ابتلاع المواد الملوثة (ليو وآخرون، ٢٠١٥). الأكثر شيوعا الأغذية الملوثة هي الآيس كريم والبيض والمحار واللحوم غير مطبوخة والفواكه والخضروات الخام، والمياه الملوثة وهناك ميزة كبيرة من الأساليب الجزيئية هو انخفاض الوقت للكشف. وتسمح مخرجات التشخيص الأآثر سرعة بالتحقيقات الوبائية السابقة وتدخلات مكافحة العدوى (شورمان وآخرون، ٢٠٠٧). تفاعل البوليميراز المتسلسل (ير) هو تقنية قوية جدا في البيولوجيا الجزيئية ويستخدم على نطاق واسع اليوم لعدد متزايد من التطبيقات مثل في التشخيص السريري، في تحديد الأفراد تسلسل الحمض النووي هو عملية تحديد النظام الدقيق من النيوكليوتيدات داخل الحمض النووي مركب. وهو يتضمن أي طريقة أو تقنية تستخدم لتحديد ترتيب القواعد الأربعة - الأدينين، الغوانين، السيتوزين، والثيمين - في حبلا من الحمض النووي