ABSTRACT

Salmonella is considered to be one of the most important causative agents which infect animal causing great mortalities and various morbidity changes. Avian salmonellosis is a large group of diseases of poultry caused by the genus *Salmonella*. Twelve strains of *Salmonella* (10 chickens and 2 ducks) isolated from poultry flocks in different geographical areas and these isolates were serotyped as Typhimurium. All strains in this study were characterized by phenotypic and genotypic methods to compare the usefulness of the methods in epidemiological studies. The obtained results, the twelve local isolates of *S.*Typhimurium were sensitive to ten different antibiotics by percentage 91.7%, 83.3%, 75% and 50% .While 100%, 83.3% and 66.7% of the isolates were resistant to this antibiotics. All isolates could be classified as either invasive or cytotoxic according different assays. The results showed that, all isolates of *S.*T. were able to invade the Vero cells by different percentage (33.3% have high invasion capability, 41.7% have moderate capability and 25% have low capability) and the isolates with high invasiveness capability exhibited high epithelial cell cytotoxicity (ranged from 49% to 80%). Also, all strains were able to adhere to Vero cells by different degrees (75% of strains have high adherence capability while 25% of them have moderate capability). The molecular characterization of Egyptian isolates were performed using sequence analysis of invA whole gene of most invasive strain that amplified by PCR technique by specific synthetized primers and all of *S.*T. strains have invA gene at specific molecular size (2058 bp). Sequencing of invA gene of the local isolate has been done for characterization and to detect the similarity and differences between it and the reference strains (isolates) all over the world. The nucleotide sequence of the highly virulence Egyptian isolate collected in 2014 was determined and encoding a 685 amino acid polypeptide then compared with invA gene of *S.*T. published sequences on Genbank. The results of the homology percentage of nucleotide and amino acid sequence leading us to say that the Egyptian *S.*Typhimurium strain has high similarity with other published *S.*Typhimurium strains (~99%) which use in antigen or vaccine production, so this local strain can be used in vaccine production in the future instead of other international strains.

Key words: *Salmonella* Typhimurium, *S.*T., Invasion, Adherence, Cytotoxicity, Sequencing.

INTRODUCTION

Salmonella is the most important causative agents which infects animal causing great mortalities and various morbidity changes and considered as the most important zoonotic diseases in the world espically *S.*enterica ***(UK Ministry of Agriculture, Fisheries and Food, 1983; UK Public Health Laboratory Service, 1984 and Wall et al., 1995)***. Avian salmonellosis is a large group of acute and chronic diseases of poultry caused by the genus *Salmonella*. Poultry and poultry products are identified as important source of *Salmonella* that cause human illness ***(Snoeyenbos and Williams, 1991 and Tietjen and Fung, 1995)***.

Lederberg, (2000) said that the *Salmonella* has natural habitats including animal intestines, plant, soil and water. The major characteristics of Enterobacteriacea (or Enterobacteria) are Gram- negative, rod-shaped bacilli bacteria that can grow aerobically and anaerobically, produce a catalase but not oxidase, ferment glucose not lactose, hydrogen sulphide producer, usually motile by peritrchous flagella and non-spore-forming.

The major concern for the industry and public health are the rapidity, cost effective and automated diagnosis of food

borne pathogens. So, that prevention of *Salmonella* infection is important for poultry health and for food processing industries (***Lim et al.; 2003***).

Recently, many factors and a complex of mechanisms are being unfolded regarding the expression of *Salmonella* virulence. Most of the virulence genes are encoded within *Salmonella* pathogenicity islands (SPIs) located at centisome 63 of its chromosome as units of large cassettes and some operons (***Marcus et al., 2000***). SPI-1 encodes the type III secretion apparatus (TTSS or T3SS) with more than 20 effectors/regulatory proteins that modify host cell signaling pathways. These secreted effectors proteins are clusters of chromosomal virulence genes found only within the genus *Salmonella (**Amavisit et al., 2003**)*. Phylogenetic analyses of the SPI-1 genes suggest that *Salmonella* has acquired this region by horizontal gene transfer from another microorganism. Nucleotide sequence analyses revealed that SPI-1 contains at least 29 genes (***Hardt et al., 1998***).

Salmonella species adhere to and invade a number of different mammalian cell lines, such as Vero cells. The insights into the molecular and genetic bases of *Salmonella* virulence provided by these studies are believed to be highly

relevant to the understanding of the natural infection process. Several laboratories have identified genetic loci involved in the entry process of *Salmonella* spp. into host cells such as Inv-locus (***Suarez and Russman, 1998***).

Salmonella Typhimurium has long served as a model organism for genetic studies, and a wide variety of classical and molecular genetic tools exist for the identification and characterization of potential *Salmonella* virulence genes. In addition, the availability of in vitro tissue culture and small animal models of infection facilitates study of complex interactions between host and pathogen during various stages of infection (***Ohi and Miller, 2001***). Many diverse bacterial pathogens share common mechanisms in terms of their abilities to adhere, invade and cause damage to host cells and tissues (cytotoxicity) (***Finaly and Falkow, 1997***).

The molecular approach to microbial pathogenesis has resulted in an impressive amount of data on bacterial virulence genes. Bacterial genome sequences rapidly add candidate virulence genes to electronic databases. The interpretation of this overwhelming information is obscured because every gene involved in pathogenicity is called a virulence gene, regardless of its function in the complex

process of virulence. A refined definition of virulence genes is proposed in which the function of the gene in the virulence process is incorporated. With the development of molecular biological techniques, it became possible to identify the genes encoding those factors responsible for virulence. This resulted in molecular microbiology, in which the role and function of specific genes (and the factors they encode) in bacterial virulence was the subject of investigation. In the beginning of molecular microbiology, genes were identified that encoded virulence factors of known reputation and these were used as probes to find analogs in other organisms (***Trudy and Gaastra, 2001***).

A more through comprehension of the common themes in microbial pathogenicity is essential to understanding the molecular mechanisms of microbial virulence and to the development of novel vaccines and other therapeutic agents for the treatment and prevention of infectious diseases ***(George et al., 2005)***.

The price and time for whole genome sequencing will soon be in the same range as the traditional typing methods mentioned above. Genome sequencing can be a powerful method in epidemiological and evolutionary investigations

(***Pallen et al., 2010 and Gardy et al., 2011)***. This requires standard procedures for identifying variation and for analyzing similarities and differences. Conserved genes are present across bacterial genomes of the same species (or genus). A fraction of these genes those conserved in all (or most) of the genomes of a given bacterial taxonomic group is called the 'core-genome' of that group. The core-genome can be identified either within a genus or species (***Malorny, 2011***) and can be used to identify the variable genes in a given genome (***Adékambi et al., 2011***). In addition, the conserved genes in general appear to evolve more slowly, and can be used for determining relationships among bacterial isolates ***(Urwin and Maiden, 2003***).

AIM OF THE WORK

Understanding of the common themes in microbial pathogenicity is essential to recognize the microbial virulence in order to develop novel vaccines and other therapeutic agents for the treatment and prevention of infectious diseases.

The aim of this study is to investigate the pathogenicity of different isolates of *Salmonella* Typhimurium on cell culture to study the different virulence factors such as: invasion, adherence and cytotoxicity.

Further studies will be carried on the most invasive isolate with special approaches (of molecular and bioinformatic studies) to the role of invA gene in invasion through the isolation of invA gene and study whether or not the invA gene of local isolates is conserved with standard *Salmonella* Typhimurium strains and could be used for the preparation of *Salmonella* antigens and vaccines from local isolate(s) in the future.

REVIEW OF LITERATURE

1.1. Recovery of Salmonella in poultry

Barrosamad and Martins (1985) recovered (101) isolates from poultry farms which included 23 of *S.*Typhimurium, 19 of *S.*Typhimurium *var* Copenhagen, 26 of *S.enteritidis*, 10 of *S.berta* and 7 of *S.havana*. Also, ***Rudy, (1985)*** identified *Salmonella* from internal organs of broilers with incidence of 38% from faeces and 22% from bedding (litter) samples. The commonest serovars were *S.*Typhimurium, *S.*Gallinarum and *S.*Enteritidis. While, 53% of 2603 isolates of *Salmonellae* belonging to 50 serovars was reported by ***Schellner, (1985)*** as *S.*Typhimurium, 18.5% *S.dublin* and 7.9% *S.tennessee*.

The incidence of *Salmonella* in chicken meat and turkey meat in Egypt were studied by ***Safwat et al. (1985)***. The incidence was 9% in chicken meat and 3.4% in turkey meat. They demonstrated a variable incidence of *Salmonella* according to the source of importation so the incidence from: France 13.4%, Israel 11.5%, Denmark 9.7%, USA 6.8%, West Germany 5%, Brazil 3.4%; serotypes were identified as *S.*Typhimurium showing the predominant serotype of isolation, *S.heidelberg, S.sandiago, S.agona, S.saintpaul, S.reading,*

S.ohio, S.brandenburg, S.infantis, S.virchow, S.colindale, S.munchen, S.newports, S.kentuchey, S.sofia, S.anatum, S.london, S.farchan, S.senttenberg, S.kaksony and *S.cannis*. Five serovars from 2240 poultry carcasses examined in Accra, Ghana, as *S.anatum, S.birkenhead, S.*Typhimurium*, S.poona* and *S.wippra* ***(Boachie, 1986)***.

A survey has been made by ***Vasa (1985)*** in the National Veterinary Institute, Finland, between 1960 up to 1980. *S.Infantis* accounted 2918 out of 3539 isolates, while *S.*Typhimurium accounted 296 isolates and 16 other serotypes were isolated in small numbers. A single hatchery might have been the source of *S.Infantis* infection.

Ianieri et al. (1986) isolated *S.Gallinarum Pullorum* from fecal samples (4%0) from traditional poultry farms in southern Italy.

A total of 14424 isolates of *Salmonella* (5555 isolates from man , 877 from cattle and buffaloes, 557 from sheep, 363 from pigs and 7072 from chickens) were serotyped by ***Murray et al.,(1998)*** who saied that *S.Dublin*, *S.*Typhimurium*, S.Derby and S.Sofia* were the predominant isolates.

Willinger et al. (1986) identified 19 *Salmonella* serotypes among poultry farms, 6 serotypes were detected in feed (*S.heidelberg, S.*Typhimurium*, S.duisburg, S.newport,*

S.saintpoul and *S.drypool*), while *S.*Typhimurium and *S.infantis* were present in poultry abattoirs.

From 20 outbreaks of *Salmonella* in poultry farms in New Caledonia, ***Desoutter (1986)*** detected *S.*Typhimurium in 50% of cases, *S.London* and *S.Muenchen* in 20% (each) and *S.Arizona* species in 10%. While *S.Gallinarum-Pullorum* has never been isolated.

Abd-Allah (1991) revealed that the incidence of *Salmonella* in broiler and parent chickens, in El-Fayoum governorate, was 6.4%. The isolates were belonging to group D1 (51.5%), group B (39.4%) and group C2 (9.1%). The most common serotypes were *S.Gallinarum-Pullorum, S.*Typhimurium, *S.Enteritidis*, *S.Dublin* and *S.Reading*.

Refai et al. (1992) recovered *S.*Typhimurium, *S.Rissen, S.Blockley, S.Newport, S.Kottbus and S.Virchaw* from poultry feeds with recovery rate 2%.

After identification of 15 different serovars from the ovaries of commercial layer hens at time of slaughter. *S.heidelberg* was the most predominant serovar (56.5%), followed by *S.Agona, S.Soranienberg*, *S.Mbandaka, S.Kentucky, S.Montevideo, S.London, S.*Typhimurium, *S.Infantis, S.Schwarzengrund, S.Ohio, S.Cerro and S.Anatum. S.Enteritidis*

phage type "23" was recorded from only one of the flocks ***(Barnhart et al., 1992)***. In 3700 pooled caecal samples from laying hens the incidence of *Salmonella* was 65.4%, but only 6 isolates were serotyped as *S.enteritidis* ***(Waltman et al., 1993)***. The presence of *S.enteritidis* in 2 out of 351 flocks of hens which produce hatching eggs were recovered by ***Ebel et al. (1994)***. *S.enteritidis* accounted 33.5% of human cases of salmonellosis.

A survey in large number of samples from different domestic birds and its environmental surroundings in El-Fayoum governorate were carried out by ***Abd-Allah; (1995)*** for detection of *Salmonella*. Serological typing of the 25 *Salmonella* isolates revealed that, 10 isolates were *S.enteritidis* (40%), 6 *S.*Typhimurium (24%), 4 *S.montevideo* (16%), 3 *S.gallinarum pullorum* (12%) and one *S.california* and *S.newport* (4% each).

Oh and Choi, (1996) isolated 42 *Salmonella* strains from 1577 caecal samples of chicks, the serotypes were: *S.typhimurium* (10), *S.*Typhimurium *var* Copenhagen (5), *S.infantis* (4), *S.thompson* (3) and 20 were untypable. The most frequent isolates from commercial turkey flocks were *S.newport* (34.6%) and *S.reading* (30.3%) followed by *S.bredney* (10.6%), *S.enteritidis* phage type 8 which was

detected for only a short period (5 weeks) in one flock . A survey among 39 poultry flocks suspected of being infected with *Salmonella* was carried out by ***Jindal et al. (1999)*** who found the *Salmonella* infection accounted 5% of the total disease outbreaks 111 poultry, with overall morbidity and mortality rates of 14.22 and 12.12% respectively.

The 231 strains of *S.*Typhimurium phage type DT104 of animal origin isolated from geese, turkeys, poultry and pig ***(Szmolleny et al., 2000)***. While, *Salmonella enteric* subsp. *enteric* serovar *enteritidis* recovered from laying fowls ***(Carli et al., 2001)***.

The prevalence of *Salmonella* serovars among Danish turkeys between 1995 and 2000 were detected by ***Pedersen et al. (2002)***, the most five prevalent serotypes which accounted for 58.5% of the isolates were *S.heidelberg, S.agona, S.derby, S.muenster* and *S.anatum*.

(Molla and Mesfin; 2003) reported *Salmonella* in chicken meat (15.4%), liver (34.5%), and heart (23.7%), identified of which *S.*braenderup which was the most frequent followed by *S.* Typhimurium.

Salmonella are widespread in humans and animals. The non-typhoid *Salmonellae* are an important cause of

bacterial gastroenteritis in industrialized countries. In the Netherlands, the estimated incidence of salmonellosis is three cases per 1000 inhabitants per year ***(Van den Brandhof et al., 2003)***.

Guerin et al., (2005) who tested the samples included chicken carcasses; chicken parts and processed chicken products the *S.*Typhimurium*, S.heidelberg, S.hadar, S.kentucky* and *S.thompson* were the most frequently isolated serovars. Over 90% of the *S.heidelberg, S.hadar, S.kentucky* and *S.thompson* were isolated from chickens.

10 serotypes of *Salmonella* from cloacal swabs obtained from 100 duck farms in Taiwan were serotyped by ***Tsai and Hsiang, (2005)*** as *S.potsdam* (31.9% of isolates), *S.dusseldorf* (18.7%), *S.indiana* (14.3%), *S.*Typhimurium (7.7%), *S.hadar* (5.5%), *S.newport* (4.4%), *S.derby* (4.4%), *S.montevideo* (2.2%), *S.schwarzengrund* (2.2%) and *S.asinnine* (1.1%).The *S.enterica* subsp. *enterica* serovar *agona* plays an important role in Brazil as causative agent of salmonellosis in food-producing animals in pigs and poultry as well as in humans ***(Michael et al., 2006)***.

El-Zeedy et al. (2007) examined a total of 620 egg samples from different poultry species (chickens, ducks and

ostriches) and 1615 poultry samples (chicken, ducks, pigeons, quails, turkeys and ostriches) for *Salmonella* infection in Egypt. Twelve *Salmonella* isolates were obtained from egg samples and 67 isolates from poultry samples and were serotyped into *S.enteritidis, S.*Typhimurium, *S.rubislaw, S.infantis, S.montevideo, S.cerro, S.virginia, S.agona, S.poona, S.derby, S.kentucky* and *S.sandiago.*

Abdellah et al., (2009) reported *Salmonella* contamination in chicken meat and giblets, 4 different serotypes were identified of which *S.* Typhimurium (40.35%) was the most frequent. *Salmonella* isolates found at level of 13.88%, 11.11% and 6.25% in chicken gizzard, liver, and breast, respectively.

The prevalence of *Salmonella* and antimicrobial resistant *Salmonella* were compared by ***Alali et al., (2010)***, as investigate the distribution of this pathogen in organic and conventional broiler poultry farms in North Carolina. All samples were analyzed for the presence of *Salmonella* using selective enrichment techniques. *Salmonella* prevalence in fecal samples was 5.6% and 38.8% from organic and conventional farms, respectively. From feed, 5.0% and 27.5% of the samples were positive for *Salmonella* from organic and conventional farms, respectively. None of the

water samples were positive for *Salmonella*. While in ***(2012); Alali, et al.*** reported *Salmonella* prevalence of 27% in broiler chicken meat in Russia Federation.

Rabie et al., (2012) reported the prevalence of the genetic types and serotypes of *Salmonella* among broiler chickens, raw chicken's meat and patients suffer from food poisoning signs in Toukh, Egypt and foundthat, 14%, 4% and 10% in chickens, raw chicken's meat and patients respectively. The *Salmonella* Isolates were serologically identified as 58.33% and 41.66% *S.Enteritidis and S.*Typhimurium.

Moussa et al., (2013) surveyed the occurrence of *Salmonella* serovars (between 2010 to 2012) and the samples were collected from 1075 of poultry, cattle and sheep and these samples were obtained from EL-Basateen slaughter house and private farms. The most common serovars were *S.*Typhimurium*, S.enteritidis, S.kentucky, S.arizona.*

In the US ***Andino and Hanning, (2015)*** reported the data from foodborne outbreaks related to human illness and showed that serovar *Enteritidis* was the most frequently isolated followed by Typhimurium, *Newport, Heidelberg*, and *Montevideo* by percentage 27%,14%,7%,10% and 3%. The food vehicles associated with this serovars including