Studies on virulence characters of *Salmonella Typhimurium* isolated from animal and human.

Khalilia A. El-Taib

Microbiology Unit, Suez Canal university Hospitals, Egypt
dr. khalilia11@yahoo.com

Abstract: The virulence properties of *Salmonella* enterica serovar *Typhimurium* that were isolated from human, cow calves and lambs were determined by using adherence assay , Congo red binding test, production of hemolysin, and HEp2 cell invasion test. The isolates were tested also for antibiotic sensitivity using disc diffusion method . PCR was involved for determine the presence of virulence invA gene that is responsible for invasion property of *Salmonella* strains. The studies was indicated that a large portion of the isolates of *Salmonella Typhimurium* were binding congo red and hemolysin production. IT was proved that *Salmonella Typhimurium* has adhesion and invasion properties which were responsible for pathogenesis. All isolates amplified by PCR were invA gene positive.

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1. Introduction

Salmonellae are one of the most important microorganisms that cause disease in man and animals and among the most common causes implicated in outbreaks of food born infectious disease around the world, **(Abou-Zeed et al., 2000)**. Animals are mainly infected through feed, drinking water or environmental sources. The risk of *Salmonella* infection has been high by the globalization of trade in food, feed and live animal and changes in production, processing and handling of foods.

Salmonellae have a wide host range, including, human, animals and birds **(Douce et al., 1991).** Salmonellae cause acute and chronic enteritis, septicemia, abortion, poly-arthritis, nervous manifestation and death **(Gorman and Adley 2004).** *Salmonellae* produce a variety of putative virulence determinants, including adhesions, invasion, fimbriae, exotoxin and endotoxin. **(Jones et al., 1982).**

Virulence of microorganism is associated with the capacity to attach and colonize at the site of infection, with subsequent damage to the host and is promoted by aggregation that interfere with the host defense **(Abou-zeed et al., 2000 and Pasquaii et al., 2004).**

We have recently cloned a group of genes (invA), that allows *S. Typhimurium* to enter cultured epithelial cells. The invA genes are arranged in the same transcriptional unit. Virulent strains of *S. Typhimurium* carrying defined mutations in invA had higher 50% lethal doses than their parent strains when administered orally to mice and were deficient in their ability to colonize Peyer's patches and the small intestinal wall. In contrast, invA mutants were fully virulent, when administered intraperitoneally, suggesting that the invA genes are only needed for the display of virulence when *S. Typhimurium* is administered by the natural route of entry **(Jorge and Roy-Curtiss, 1991).** In addition, conduction with transcriptional and translational fusions of reporter genes to invA had established that the expression of the invA genes is regulated by changes in DNA **(Tanaka, K, et al 2004).**

The isolation and identification of *Salmonellae* by traditional methods is time-consuming and laborious. The polymerase chain reaction (PCR) provides a way of overcoming these difficulties and allows amplification of the target DNA **(Murugkar et al., 2003).** The aim of this study was to determine serological and virulence patterns of *Salmonella Typhimurium* isolated from cow calves, lambs and human. All isolates were tested for antimicrobial drug susceptibility. Moreover, to determine the genotypic characteristics including the presence of virulence invA gene at different isolates.

2. Material and Methods

Isolation and identification of *Salmonellae:*

A total of 204 fecal samples from cow calves lambs and human were cultured in selenit F broth and incubated at 37°C for 18 hours, a loopful from inoculated broth was streaked onto the surface S.S. agar plates, then incubated at 37°C for hours. Suspected colonies were identified culturally and biochemically according to and serologically by slide agglutination test using *Salmonella Typhimurium* antisera.

Detection of virulence factors:

1. Congo red (C.R.) binding activity (Agenta et al; 1997):

Salmonella strains were cultured onto Congo medium. CR-positive *Salmonella* isolates were identified by the appearance of red colonies. The reaction was best seen after 24 hrs incubation at 37°C, followed by an addition days at room temperature. CR-negative *Salmonella* colonies did not bind the dye (white colonies).

2. Detection of hemolysin (Jones et al; 1982 and Agenta et al ;1997):

B-hemolysin:

Salmonella isolates were inoculated into blood agar plates containing unwashed sheep blood 5%, after 24 hours of incubation at 37°C , positive B-hemolysin production was indicated by clear zone of hemolysis.

3. Adherence assay (Douce et at 1991):

Loopful of overnight bacterial cultures in peptone water (containing 1 % D-mannose) were inoculated into cover slips 24 well plate, which had been seeded with 5 X 1 O HEP-2 cells 48 hr. Cultures were incubated at 37°C for 3 hrs. The cells were washed. Fresh Hanks 199 was added and then the cells were incubated for further 3 hrs. Then cells were fixed with 3% formalin and stained with Geimsa solution. The adhesion was determined by light microscope covering the whole slide. Bacteria were recorded as adhesive if a cluster of at least 10 bacteria adhered per HEP-2cell.

4. Invasion assays (Dutjus et el , 1998):

Loopful of bacterial culture in peptone water were incubated with HEP-2 cells for 2-3hrs to allow attachment and penetration of epithelial cells.. Gentamicin (which is unable to penetrate mammalian cells) was added to eliminate extracellular bacteria. The cell sheet was washed, fixed and stained by Giemsa.

In vitro susceptibility of Salmonella isolates to various antimicrobial agents:

Antimicrobial drug sensitivities were determined for each *Salmonella* isolate using disc diffusion method and commercial discs **(Djukeren et al., 2003).**

Extraction of DNA from bacteria:

The strains were routinely grown for 24 hours at 37°C in 5m1 of Luria broth medium in Loose-top culture tubes with aeration at 90 rpm in shaker incubator. The organisms were pelleted by centrifugation at 8000 X g for 10 minutes, washed twice with l0ml of phosphate buffer saline and resuspended in 2ml TE buffer (10mM Tris, 1mM ethylene-diamine tetra-acetic acid (EDTA. pH 7.6). The bacteria were lysed with sodium dodecyl sulphate at the final concentration of I % and were incubated at 37°C for 1 hour. The mixture was then treated with proteinase K (20mg/ml) and incubated at 37°C for 1 hour. The chromosomal DNA was then extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25 24: 1). The DNA was precipitated with 1/10 volume of 3M sodium acetate and 2 volumes of absolute ethanol and incubated overnight at-20°C. the precipitated DNA was pelleted by centrifugation at 8000 Xg at 4°C for 10 minutes and resuspended in 500ml TE buffer. **(Amin and mazhar, 1997).**

Oligonucleotide primers:

Two pairs of oligonucleotide primers specific for salmonella invA gene were used for the PCR methods. This primer was predicted to yield a 521 bp product (Jorge and curtiss 1991).

The oligonucleotide primers specific for salmonella invA gene:

The invA sense primer: 5'-TTG TTA CGG CTA
The TGA CCA-3'
The invA antisense primer: 5' CTG ACT GCT
ACC TTG CTG ATG-3'

DNA amplification:

DNA amplification was performed according to **Abou-zeed et al. (2000)** in a volume 50j.d containing 10 mM Tris Hcl pH 8.3, 50 mM Kcl, 1.5 mM MgCI2, 50 ng template DNA, 200j.M of each of four deoxyribonucleotide triphosphates dATP, dGTP, dCTP and dTTP, 20 PM of each oligonucleotide primers, and 1.5 MO Taq-DNA polymerase enzyme. Samples were subjected to heating for 5 minutes at 94°C then using 35 cycles of denaturation at 93°C for 1 minute, annealing for I minute at 52°C and extension for 2 minutes at 72°C. the last cycle was followed by a final 10 minutes extension at 72°C.

Electrophoretic detection of PCR products:

The PCR products were visualized by gel electrophoresis. Samples of final PCR product were mixed with loading dye and loaded onto a 1.5% agarose gel and subjected to electrophoresis for 1 hour at two V. in LX TBE buffer **(Tris HcL Boric acid, EDTA)(Murugkar et al., 2003) .**

3. Results:

The table 1 showed that the incidence of *Salmonella Typhimurium* was (4.76%) and (6.45%) in apparently healthy calves and lambs while in

diarrheic calves, lambs and human it was (16.07%), (15.15) and (9.52) respectively.

The typing of the tested strains isolated from diarrheic calves, lamb, and human as a confirmatory step is shown in Table (2); all examined isolates recovered from diarrheic and apparently healthy animals and human were bounded with Congo red dye giving red colonies 18, isolates (81.81 %) and for B hemolysin producer 17 isolates (7 1.27%).

Results in Table (3) revealed that is (68. 18%) 1 *Salmonella* isolates could adhere to HEP-2 cells and invasion 13 isolates invaded the cells (59.09%).

Results illustrated in Tables (4and 5) revealed that the in vitro sensitivity of Salmonella isolates against to antimicrobial agents. The examined isolates were proven to be sensitive to Ofloxacin, Gentamicin, Ciprocin, Nitrofurantoin and Chloramphenicol while Ciprocin, Gentamicin and Ofloxacin complete sensitive in human.

The attachment and penetration effect of the isolates were observed in fig. 1 which showed cytopathic effect of the cells .

The optimal reaction condition for amplifying a template DNA was optimized in relation to different factors such as: primer structure, magnesium ion concentration, annealing temperature and DNA polymerase enzyme. The effect of these factors was qualitatively evaluated by determination of the PCR amplification products fractionated on agarose gel and visualized under U.V. light after staining with ethidium bromide. The obtained data indicated that the optimal concentration of magnesium ion in the reaction was 1.5 JM, Taq polymerase enzyme concentration was 1.5JM, primer concentration was 10 PM and optimal annealing temperature was 52°C. six isolates were examined by PCR to determine the virulence invA gene. All isolates were positive for invA gene sequences as indicated by the size of the PCR product in agarose gels (approximately 521 bp) (Fig. 2).

Table (1): Incidence of *Salmonella Typhimurium* isolated from animals and human.

Species	Total number of examined samples	N. of isolated *Salmonella Typhimurium* from animal and human	Apparently Normal		diarrheic animals and Human	
			No. of fecal samples	No. of positive samples	No. of diarrheic fecal samples	No. of positive samples
cow calves	98	11 (11.22%)	42	2 (4.76)	56	9 (16.07)
Lambs	64	7 (10.93%)	31	2 (6.46)	33	5 (15. 15)
Human	42	4 (9.52%)	-	-	42	4 (9.52)
Total	204	22 (10.78%)	73	4 (5.47)	131	18 (13.74%)

Table (2) Biological characteristics *Salmonella Typhimurium* strains isolated from cow calves, lambs and human.

Source of samples	No. of isolates	Congo Red assay		B- haemolysin	
		No.	%	No.	%
Cow calves	11	9	81.81	9	81.81
Lambs	7	5	71.42	5	71.42
Human	4	4	100%	3	75.00
Total	22	18	81.81	17	77.27

Table (3): Adherence and Invasion properties of different Serogroups isolated from cow calves, lambs and human.

Serogroup	No. of isolates	Adherence assay		Invasion assay	
		Positive		Positive	
		No.	%	No.	%
Cow calves	11	8	72.72	7	63.63
Lambs	7	5	71.42	4	57.14
Human	4	2	50.00	2	50.00
Total	22	15	68.18	13	59.09

Table (4): Antimicrobial susceptibility pattern of 18 isolates of *Salmonella* isolated from animals.

Antimicrobial agents	Conc. (µg)	Antimicrobial Susceptibility		
		Sensitive	Intermediate	Resistant
Ampicillin	40µg/ml	9/18(50%)	2/18(11.11%)	7/18(38.88%)
Chloramphenicol	30µg	15/18(83.33%)	1/18 (5.55%)	2/18(11.11%)
Ciprocin	5 µg	16/18(88.88%)	-	2/18(11.11%)
Erythromycin	15µg	8/18 (44.44%)	2/18(11.11%)	8/18(44.44%)
Gentamicin	10µg	17/18(94.45%)	-	1/18(5.55 %)
Nalidixic acid	30 µg	10/18(55.55%)	3/18(16.66%)	5/18(27.77%)
Nitrofurantoin	300 µg	16/18(88.88%)	-	2/18(11.11%)
Ofloxacin	5 µg	18/18 (100%)	-	-
Streptomycin	10 µg	9/18 (50%)	5/18(27.27%)	4/18(22.22%)
Tetracycline	30 µg	1/18 (5.55%)	3/18(16.66%)	14/18(77.77%)

Table (5): Antimicrobial susceptibility pattern of 4 isolates of *Salmonella* isolated from human.

Antimicrobial agents	Conc. (µg)	Antimicrobial Susceptibility		
		Sensitive	Intermediate	Resistant
Ampicillin	40µg/ml	3/4 (25%)	-	¼ (25%)
Chloramphenicol	30µg	1/4 (25%)	1/4 (25%)	2/4 (50%)
Ciprocin	5 µg	4/4 (100%)	-	-
Erythromycin	15µg	-	1/4 (25%)	3/4 (75%)
Gentamicin	10µg	4/4 (100%)	-	-
Nalidixic acid	30 µg	-	2/4 (50%)	2/4 (50%)
Nitrofurantion	300 µg	-	1/4 (25%)	3/4 (75%)
Ofloxacin	5 µg	4/4 (100%)	-	-
Streptomycin	10 µg	-	-	4/4 (100%)
Tetracycline	30 µg	-	-	4/4 (100%)

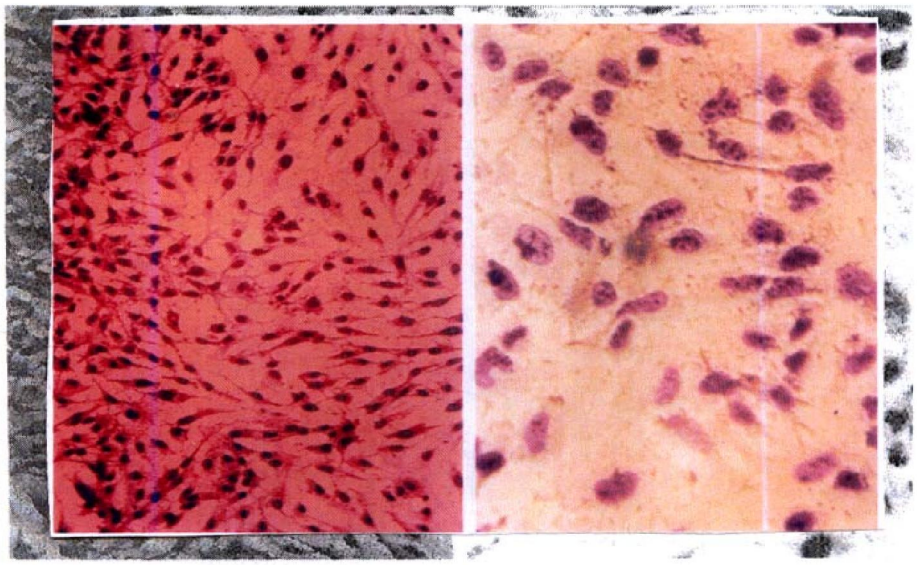

Fig. (1): HEp-2 cell invasion. A= Control negative. B= positive invasion strain.

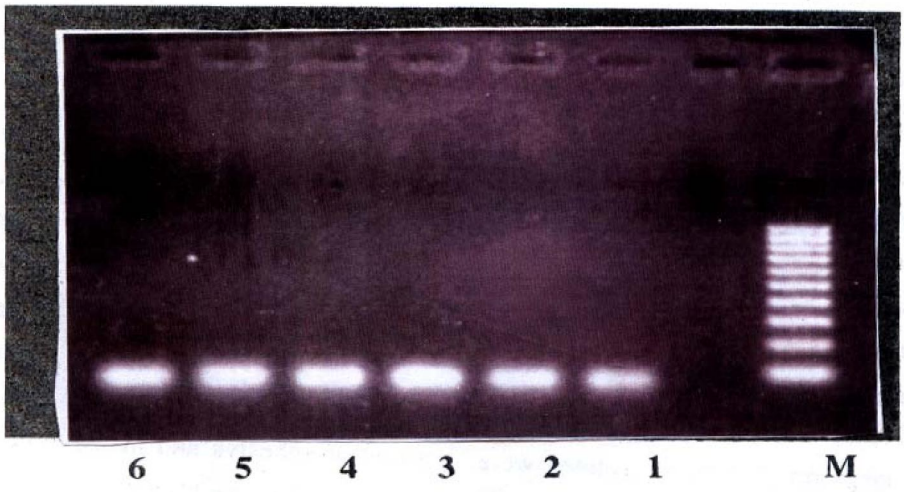

Fig. (2): Analysis of PCR products of invA gene of salmonella isolates of 15% agarose gel electrophoresis Lane M. l00bp ladder; Lane I and 2 Salmonella enterica serovar Typhimurium Isolates for cow calves. Lane 3 and 4 Salmonella enterica serovar Typhimurium Isolates from lambs. Lane 5 and 6 Salmonella enterica serovar Typhimurium Isolates from human.

4. **Discussion:**

Salmonellae are thought to be the major pathogens leading to serious economic losses in animal, industry and human health. The highest incidence of *Salmonella Typhimurium* was (11.22% recovered from calves, followed by lambs and human (10.93%) and (9.52%). It is worthly to denote, that our result *S. Typhimurium* agreed with that reported in human **(Obil et al., 1997)** and calves and lambs. also these result agree with who reported such the incidence of *Salmonella* in lambs varied from o to up to 45**%. (Hemmat and Salemi ,1994 and Khalil, 1988) .**

Identification of virulence determinants of *Salmonellae* isolates had led to better understanding

of pathogenesis of diarrheal disease caused by them and providing a new dimension to their diagnosis.

The results illustrated in table (1) showed that all isolates recovered from animals and human bind Congo red. Pathogenic *Salmonella* have evolved some unique cellular products associated with virulence of organism. The results indicated that a large proportion of isolates were hemolysin producer and this could be used as phenotypic marker or virulence factor. Even though strains which elaborated hemolysin were frequently associated with diarrhea and played critical role in extra-intestinal infection, there was no evidence to suggest that the elaboration of hemolysin increases the potential for causing diarrhea. in addition, it is important to note that the adherence and invasion of micro-organism to HEP-cells is controlled with the invasion of the gastrointestinal mucosa of the organisms which is an critical step in pathogenesis caused of *Salmonella* microorganism **(Murugkar et al., 2003).**

The ability to adhere to intestinal epithelial cells was an important virulence factor. Adhesion would allow organisms to overcome the disadvantages of living in constantly moving environment. The epithelial surface was likely to provide a more stable environment than the lumen of intestine and the bacteria would be in close approximately to nutrient transport. The results illustrated in table (3) agree with **(Agenta et al., 1997),** who recorded adhesion at the ranges of 66.77 and 88.1% among Salmonella recovered from human and calf. In vitro invasion test revealed that 13 (59.09%) isolates were positive**. Khalil (1988**) recorded that invasion was at ranges of (36.4%- 50%) according to sources of isolates. However, no relationship could, be found between adherence, and invasivness. The invasion of HEp-2 cells by *Salmonella* serórvars is a useful model for study of adhesive and invasive properties of this pathogen. The penetration of HEp-2 cells appears to result from endocytosis of the bacteria by the animal cells **(Jones et al., 1982).**

All tested serovars were highly sensitive of *Salmonella* to 10 antimicrobial agents. The examined isolates were proved to be highly sensitive to ofloxacin, Gentamicin, Ciprocin, Nitrofurantoin and Chloramphenicol for animals isolates while Ciprocin, Gentamicin, Ofloxacin complete sensitive to human isolates **(Wedel et al., 2005).**

The PCR is a highly accurate method which makes it possible to detect nucleic acid amplification products. The results can be obtained rapidly so that they can be used not only to support bacteriological investigation but also to make the result more reliable **(Galan and Curtiss 1991).**

In the present study, a PCR was used to amplify *Salmonella* specific target DNA sequences. A new set of oligonucleotides primer taken from the invA gene was used for amplification to detect and identify *Salmonella* serovares. The primers are different from those suggested in previous studies of **(Baumler et al., 1997).** The difference consisted in the sequence and in the annealing sites on the nucleotide sequence of the invA gene.

The most suitable condition for DNA amplification was magnesium ion concentration, DNA polymerase enzyme concentration and annealing temperature. These agreed with **(Tanaka et al., 2004)** , who mentioned that the optimal PCR condition for amplifying a template DNA can vary from one primer to another and necessary to be determined empirically.

The investigation using PCR for the presence of invA gene in this study demonstrated its presence in all examined salmonella isolates in respective of the serovar or source. This finding was consistent with previous reports (Galan and Curtiss, 1991) that established the presence of invA gene in nearly all *Salmonella* irrespective of serovar or source. The invA gene is important in the invasion of phagocytic epithelial cells and entry into the intestinal mucosa. This was confirmed by Tanaka et al., 2004 who showed that *S. Typhimuriurn* strains carrying mutation in invA genes are unable to selectively invade in the follicle- associated epithelium of murine Payer's patches the results of this study agreed with **(Murugkar et al., 2003)** who reported that all salmonellae possessed the genetic information on invasion (invA), they adhered equally well to epithelial cells, could penetrate into these and survive and multiply inside cells. Since adhesion, invasion and the ability of intracellular survival and multiplication, constitute the most virulence parameter of salmonella.

From the present studies it was concluded that *S. typhimuriurn* is highly pathogenic micro-organism affected animal production and cause serious human diseases.

The virulence effects of *S. Typhimuriurn* attributed to its abilities to adherence and attachment to the host epithelial cells, also the presence of invA gene which responsible for the invasion properties .

Our studies indicated that the incidence of *S. Typhimuriurn* infection for cow calves were higher than that of lamb and human, it may attributed to its increase susceptibility to the virulence factors.

The isolated *Salmonella* from human and animals were sharing in sensitivity for Ciprocin, Gentamicin and Ofloxacin , on the other hand, while the animal isolates were sensitive to nitrofurantoin , the human ones were resistant .

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