Bacteriological studies on *Salmonella* isolated from cattle

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Abstract

Salmonella enterica serovar Typhimurium isolates isolated from cow calves , cows and buffaloes were tested for their virulence attributes using Congo red binding test, ability to produce hemolysin, adherence assay and HEP2 cell invasion test. Moreover, antimicrobial drug sensitivity was determined. using disc diffusion method. The results showed that the incidence of *Salmonella* Typhimurium was 8.33 %, 9.0% and 7.6% in apparently healthy calves , cows and buffalos while in diarrhaeic calves, cows and buffaloes it was 22.5 %, 22.7 % and 12 % respectively. Among the examined isolates: 18 isolates, 81.8% recovered from diarrhaeic and apparently healthy cow and buffaloes were bounded with Congo red dye and for *B. hemolysin* producer were 16 isolates 72.7 %. Moreover 16 isolates 72.7% can cause adherence and 14 isolates 63.6% can invade the cells. The examined isolates from cow were proven to be sensitive to Ofloxacin 94.7%, Gentamicin 89.4%, Ciprofloxacin 84.4%, Nitrofurantoin 84.2% and Chlormphenicol 74.9% while these isolates were resistant to Erythromycin and Tetracycline by percentages 78.9%. The examined isolates from buffaloes were highly sensitive to Ciprofloxacin, Gentamicin and Ofloxacin 100% while they were resistant to Streptomycin and Tetracycline 100%.

Key words: *Salmonella Typhimurium*, *HEP2, B. hemolysin, buffaloes* .

Introduction

Salmonellosis is a bacterial disease with a rising prevalence in the cattle industry **Huston *et al.* (2002**). The most common period of infection in dairy calves is one to ten weeks of age, but can also be seen in adult dairy cows and beef cattle (**Smith *et al*., 1980** and **Hoiseth and Stocker, 1981).** Livestock mortality, treatment costs, abortion, reduced production, discarded milk and reduced consumer confidence all contribute to the effect of *salmonella* to cattle industries **.**Salmonellae are one of the most important micro-organisms that cause disease in man and animals and among the most common causes implicated in outbreaks of foodborn infectious diseases around the world (**Abou-Zeed *et al*., 2000).**

Salmonella spp. infection occurs when a susceptible animal ingests the bacteria. Dairy cattle ingest feed or water that has been contaminated with feaces from animals shedding the organism. Salmonellosis has wide spectrum manifestations in cattle. Asymptomatic, mild clinical or fulminant bacteremia/septicemia and endotoxemic infections can occur. The manifestations vary with virulence of the strain, infectious dose, and immunity of the host (**McGuirk and Peek, 2003)** .

The risk of *Salmonella* infection has been heightened by the glo-balization of trade in food, feed and live animal and changes in production, processing and handling of foods (**Van *et al*., 2006)**.

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 manifestations and death (**Gorman and Adley, 2004).**

Sever systemic sequels depend on the type of *Salmonella* serovars and host specific factors. More than 2,400 serovars exist, and are divided into three broad classes according to their host specificity (**Uzza *et al.*, 2000).** Host–restricted serovars are primarily associated with systemic disease in one host but may cause disease in a limited number of

other species (e.g., *S.* enterica Dublin in cattle and human) (**Kunze *et al*., 2008)**. Many outbreaks of *Salmonella* infections had been reported worldwide and the most frequently isolated serovars are *S.* Typhimurium ,*S.* Enteritidis , *S,* Anatum, *S.* Newport, *S.* Cerro, *S.* Montevideo, *S.* Agona and *S.* Dubin which are considered the major host adapted salmonella of cattle (**Konral *et al.*,1994**; **Veling *et al*., 2002** and **Kunze *et al.*, 2008)** and young calves are the most susceptible to infection (**Smith *et al*.,1980** and **Moussa *et al.*, 2004).**
Salmonellae produce a variety of putative virulence determinants, including adhesions, fimbriae, exotoxin and endotoxin (**Jones *et al*., 1982).**
Virulence in 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 aggressions that interfere with the host defence (**Abou-Zeed *et al*., 2000** and **Pasquali *et al.*, 2004).**
The purpose of this study is the isolation and serological identifications of Salmonellae causing enteritis in cow calves ,cows and buffaloes ,also determine the virulence factors of isolated Salmonellae. All isolates were tested for antimicrobial drug susceptibility.

Material and Methods

1- Sample collection :
A total of 162 faecal samples were collected from cow calves ,cows and buffaloes with symptoms of diarrhea, fever, dehydration, bloody feaces, emaciation, rapid breathing, foul odor of feaces, sloughing of skin from extremities . Also feacal samples were collected from apparently healthy contact animals .Samples were collected from farms in Ismallia and El-fayoum governorates ,and were transported to the laboratory on ice box.

11-**Isolation and identification of *salmonellae*:**

Feacal samples were inoculated into selenite F broth and incubated at 37°C for 18 hours, a loopful from inoculated broth were streaked onto the surface XLD agar , MacConkey agar and S.S. agar plates, then incubated at 37°C for 24-48 hours. Suspected colonies were identified microscopically and biochemically according to **Qunin *et al*., (2002**). Serological identification was done by slide agglutination test using *Salmonella Typhimurium* antisera(DENKA-SEIKEN CO) in serology unit at Animal Health Research Institute - Dokki .

111-Detection of virulence factors:

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

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

Detection of haemolysin (Tang *et al*., 1993):

Haemolysin:

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

Adherence assay (Douce *et al* 1991):

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

Invasion assays (Dinjus *et al*., 1998):

Bacterial culture in peptone water were incubated with HEP-2 cells for 2-3hrs to allow attachment and penetration of epithelial cell. Gentamicin (which is unable to penetrate

mammalian cells) was added to eliminate extracellular bacteria. The cell sheet was washed, fixed and stained by Giemsa.

1V-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 disks (**Djukeren *et al*., 2003)**. Ampicillin , Chlormphenicol , Ciprofloxacin, Erythromycin , Gentamicin, Nalidixic acid, Nitrofurantoin, Ofloxacin , Sterptomycin and Tetracycline.

Results

The results showed that the incidence of *Salmonella* Typhimurium was 8.3 % , 9.0% and 7.6% in apparently healthy calves, cows and buffalos while in diarrhaeic calves, cows and buffalos it was 22.5 %, 22.7% and 12 % respectively as shown in table (1).

The virulence factors of the tested strains isolated from diarrhaeic calves, cows and buffalos as a confirmatory step are shown in Table (2); most of examined isolates recovered from diarrhaeic and apparently healthy animals and buffalos were bounded with Congo red dye giving red colonies in 18 isolates 81.8% and for *B. hemolysin* producer 16 isolates 72.7 %.

Results in Table (3) revealed that 16 isolates 72.7% of *Salmonella* isolates could adhere to HEP-2 cells and 14 isolates, 63.6% invaded the cells. Results showed in Table (4) revealed. That in vitro sensitivity testing of *Salmonella* strains isolated from cow calves and cows against 10 antimicrobial agents. The examined isolates were proven to be sensitive to Ofloxacin 94.7%, Gentamicin 89.4%, Ciprofloxacin 84.4%, Nitrofurantoin 84.2% and Chlormphenicol 74.9% while these isolates were resistant to Erythromycin and Tetracycline by percentages of 78.9%. Results in table (5) illustrate the in vitro sensitivity of *Salmonella* isolated from buffaloes against 10 antimicrobial agents. The examined isolates were highly sensitive to Ciprofloxacin, Gentamicin and Ofloxacin (100%) while these isolates were resistant to Streptomycin and Tetracycline 100%.

Table (1). Incidence of *salmonella* typhimurium isolated from fecal samples of cow and buffaloes.

Species	Total number of examined samples	Apparently Normal		Diarrhoeic animals human		No. of isolated *Salmonella*
		No. examined samples	No.+(ve)% positive samples	No. examined	No. of +(ve)% diarrheic fecal samples	
Cow calves	76	36	3(8.3%)	40	9(22.5%)	12(15.7%)
Cow	54	22	2(9.0%)	32	5(22.7%)	7(12.9%)
buffalos	32	7	0	25	3(12%)	3(9.3%)
Total	162	65	5(7.6%)	97	17(17.5%)	22(13.5%)

Table (2). Incidence Congo red binding and haemolycin activities of *Salmonella* Typhimurium strains isolated from , cow calves , cow and buffaloes.

Source of samples	No. of isolates	Congo red assay		B-haemolysin	
		No.	%	No.	%
Cow calves	1 2	10	83.3	9	75
Cows	7	5	71.4	5	71.4
Buffaloes	3	3	100	2	66.6
Total	22	18	81.8	16	72.7

Table (3). Adherence and invasion properties of different *Salmonella* Typhimurium from cow calves, cow and buffaloes.

Serogroup	No. of isolates	Adherence assay		Invasion assay	
		Positive		Positive	
		No.	%	No.	%
Cow calves	12	9	75	8	66.6
Cows	7	5	71.4	4	57.1
buffaloes	3	2	66.6	2	66.6
Total	22	16	72.7	14	63.6

Table (4). Antimicrobial susceptibility pattern of *Salmonella* Typhimurium isolates from cows.

Antimicrobial agents	Conc. (µg)	Antimicrobial susceptibility		
		Sensitive	Intermediate	Resistant
Ampicillin	40pg/ml	10/19 (52.6%)	2/19 (10.5%)	7/19 (36.8%) %)
Chlormphenicol	30µg	15/19 (78.9%)	1/19 (5.2%)	3/19 (15.7%)
Ciprofloxacin	5 pg	16/19 (84.2%)	0	3/19 (15.7 %)
Erythromycin	15 pg	2/19 (10.5 %)	2/19 (10.5%)	15/19 (78.9%)
Gentamicin	10 pg	17/19 (89.4%)	0	2/19 (10.5%)
Nalidixic acid	30 pg	10/19 (52.6%)	3/19 (15.7%)	5/19 (26.3%)
Nitrofurantoin	300 pg	16/19 (84.2%)	0	2/19 (10.5 %)
Ofloxacin	5 pg	18/19(94.7%)	0	1/19(5.2%)
Streptomycin	10 pg	9/19 (47.3%)	6/19 (31.5 %)	4/19 (21.0 %)
Tetracycline	30 pg	1/19 (5.2%)	3/19 (15.7%)	15/19 (78.9%)

Table (5). Antimicrobial susceptibility pattern of *Salmonella* Typhimurium isolates from buffaloes.

Antimicrobial agents	Conc. (µg)	Antimicrobial susceptibility		
		Sensitive	Intermediate	Resistant
Ampicillin	40pg/ml	2/3 (66.6%)	0	1/3 (33.3%)
Chlormphenicol	30µg	1 /3 (33.3%)	2/3 (66.6%)	0
Ciprofloxacin	5 µg	3/3 (100%)	0	0
Erythromycin	15 µg	0	1/3 (33.3%)	2/3 (66.6%)
Gentamicin	10 µg	3/3 (100%)	0	0
Nalidixic acid	30 µg	0	2/3 (66.6%)	1/3 (33.3%)
Nitrofurantoin	300 µg	0	1/3 (33.3%)	2/3 (66.6%)
Ofloxacin	5 µg	3/3 (100%)	0	0
Streptomycin	10 µg	0	0	3/3 (100%)
Tetracycline	30 µg	0	0	3/3 (100%)

Discussion

Salmonellae are Gram negative facultative bacteria causes wide rang of infectious syndrome varied from mild enteritis to sever systemic infection in variety of animal host.

Salmonella infections occur worldwide in both developed and developing countries and are major contributor to morbidity and economic posses (**Antoine *et al*., 2008).** In this study the highest inci-dence of *Samonella* Typhimurium was 15.7% discovered in calves, followed by cows and buffaloes 12.9% and 9.3%. It is worthily to denote, that our result agreed with that reported in buffalos (**Obi *et al*., 1997)** and calves and cows (**Moussa *et al*., 2004**) . The obtained percentage were higher when compared with that recorded by **Cortez *et al*. (2008)** who reported that the percentage of positive *Salmonella* samples was 10%.While **Moussa *et al*., (2010)** and **Moussa *et al*. (2012)** isolate *salmonella* from cattle in an incidence of 4.4%. Our result was lower than that reported by **Ibrahim *et al*. (2008)**. Differences in prevalence may be the result of using types of variety of samples or different methods for detection of Salmonellae.

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 occurrence of distinct pathogroups could be shown within one serogroup (**Gorman and Aldey, 2004).** The results given in Table (2) showed that most isolates found in animals and buffaloes bind Congo red, which agreed with that reported by **Khalilia (2011)**. Pathogenic *Salmonella* have evolved some unique cellular products associated with virulence of organism. The results in-dicated that a large proportion of isolates were haemolysin producer and this could be used as phenotypic marker or virulence factor. Even though strains which elaborated haemolysin were frequently associated with diarrhoea and played critical role in extra-intestinal infection, there was no evidence to suggest that the elaboration of haemolysin increases the potential for causing diarrhoea. 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 a critical step in pathogensis of *Salmonella* microorganism (**Murugkar *et al*., 2003).**

The ability to adhere to intestinal epithelial cells is 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 thus 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 75%, 71.4% and 66.6% in cow calves, cows and buffaloes, respectively. The invasion of HEP-2 cells by *Salmonella* serovars is a useful model for study of adhesive .The invasive properties 66.6 %, 57.1% and 66.6% of this pathogen among *Salmonella* found in from cow calves and buffaloes respectively

Invasion test revealed that 14 63.6% isolates were positive. **Khalil (1988)** recorded that invasion was at ranges of 36.4%-50% according to sources of isolates.

The examined isolates were proved to be highly sensitive to ofloxacin, Gentamicin, Ciprofloxacin, Nitrofurantoin and Chlormphenicol while buffaloes were sensitive to Ciprofloxacin, Gentamicin. Ofloxacin was completely sensitive to buffalos isolates which agreed with **Wedel *et al*., (2005).**

From this study it is concluded that *Salmonella* is frequently associated with gastroenteritis in cow calves ,cows and buffaloes . Moreover, the variety in the number and distribution of different virulence factors among the *Salmonella* typhimurium strain suggests that within this serovar there are different pathotype potentially responsible for different clinical syndroms. Ofloxacin**,** Gentamicin and Ciprofloxacin were drugs of choice **.**

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دراسات بكتريولوجية على ميكرونب السالمونيلا المعزولة من الماشيه

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الملخص العربى

تم عزل ميكروب السالمونيلا تيفي ميويم من الحيوانات (العجول الصغيره من الابقار والجاموس) ودراسة بعض عوامل الضراوة مثل خاصية الاتحاد بصبغة الكونجو الحمراء وإفراز إنزيم تحلل الدم وخاصية إلتصاق وغزو الخلايا وأجريت إختبارات الحساسية لهذه المعزولا ت للمضادات الحيويه.

واظهرت النتائج فى هذه الدراسه ان معدلات عزل السالمونيللا تيفي ميوريوم من الحيونات السليمه ظاهريا3و8% &0و9% &6و7% من العجول الصغيره والابقار والجاموس على التوالى وكذلك من الحيونات المصابه بنسبه 5و22% &7و22% &12% من العجول الصغيره والابقار والجاموس على التوالى . ووجد ان معظم هذه المعزولات من الحيونا المصابه والسليمه ظاهريا تتحد بصبغه الكنجو الحمراء بنسبه 8و81% وكذلك افراز انزيم تحلل الدم بنسبه 7و72%. وكذلك خاصيه الالتصاق للخلايا بنسبه 6و63% .

ونتيجه اختبار الحساسيه لهذه المعزولات من الابقار كانت حساسه للاوفلاكسين 7و94% وجنتاميسين 4و89% وسبروفلوكساسين4و84% ونيتروفرانتين2و84% وكلورامفنيكول 9و74% بينماكانت هذه المعزولات مقاومه الارثرومايسين والتتراسيكلين.المعزولات من الجاموس كانت حساسه للسبروفلاكساسين والجنتاميسين والافلوكساسين بنسبه 100% بينما كانت هذه المعزولات مقاومه للستربتومايسين والتتراسيكللين بنسبه 100% .