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Detection of gene *stn* in some non-typhoidal Salmonella spp. which isolated from patients with diarrhea

S. M. Tawfiq

Department of Soil and Water, College of Agriculture, University of Salahaddin, Erbil , Kurdistan region , Iraq

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Corresponding Author:

Name: S. M. Tawfiq

E-mail:

dr.shamerantawfiq@gmail.com

Tel:

ABSTRACT

The present study was conducted to estimate the prevalence of Non-typhoidal Salmonella spp. From April 2017 to September 2017 approximately 220 samples from diarrhoeic stool of human ages between one month till above sixty years were collected in Rizkary , children's and Kirkuk hospitals to determine the prevalence *stn* gene among Non-typhoidal Salmonella spp(*S. enteritidis*). A total of 220 samples were collected, The colonial morphology, staining, microscopical examination, cultural media as well as biochemical characteristics of the isolates found out the presence (72) 32.7% out of 220 salmonella species isolates, (41) 57% were belong to NTS from 72 isolates while *S. enteritidis* were 28(68.2%) from 41 isolates of NTS. PCR assay was carried out to detect the presence of *stn* gene, (24) 85.7% isolates of *S. enteritidis* contained the gene among 41 isolates of *S. enteritidis*. 100% of isolates were susceptible to Norfloxacin, while 16.7% were sensitive to Cefotaxime, 70% of isolates appeared multidrug resistance. 32.1% infections recorded in ages above sixty,25% age less than one year.

Introduction

Nontyphoidal salmonella (NTS) or salmonellosis (food poisoning) refers to sicknesses in humans and animals with every serotypes of *Salmonella* but not including Typhi, Paratyphi (A,B,C). NTS are considered the major cause of foodborne disease especially *Salmonella* enteritidis is the most frequently isolated serovar in Europe, South America and Asia in diarrhoeic and a considerable number of deaths globally [1]. There for, it is essential to distinguish Salmonella from each other, for the purpose of ensure every pathogen and epidemiology is appropriately known [2,3]. NTS are a major reason of bacterial diarrhea worldwide, and caused gastroenteritis sickness is usually diagnosed like insensitive diarrhea, gripes with nausea due to enterotoxin excretion by bacteria, which also be frequently cytotoxic and destroy cells by means of changing the apical membrane permeability of the mucosal cells of the intestinal barrier. The sickness usually lasts four–seven days, and the most people recover with no therapy. Antimicrobial treatment are only required in severe cases such as in immuno-compromised patients or invasive infections. About 5% of patients extend bacteremia or central disease (meningitis) [4]. quantity of invasive infections and fatality are typically elevated between The old, infants, younger adults and people with efficacy of the immune system circumstances, Infection with antibiotic-resistant microbes has been coupled with a advanced danger of bloodstream infection and hospitalization [4].

The virulence of *Salmonella spp*. is associated with a combination of chromosomal and plasmid factors, many studies have identified genes that encode these factors. Some virulence factors are associated with the cellular structure of the bacteria, such as fimbriae[5,6]. One of the main functions of aggregative fimbria (agf operon) is to promote the initial interaction of the bacteria with the intestine of the host and stimulate bacterial self-aggregation, resulting in higher rates of survival .The Salmonella-encoded fimbria (sef operon) promotes a better interaction between the bacteria and the macrophages [7].

Salmonella spp. pathogenicity islands (SPI) are of critical importance for Salmonella spp. virulence, once they encode a molecular apparatus called the type III secretion system (TTSS), which is able to

inject bacterial effector proteins through bacterial and host membranes to interact with host cells[8]. The hilA gene encodes the central regulator HilA, which is necessary for the expression of the TTSS components. HilA is also required to invade epithelial cells and induce apoptosis of macrophages The protein InvA is essential for epithelial invasion and AvrA is an effector protein of the TTSS complex that contributes to the virulence of Salmonella spp. by limiting the host's inflammatory responses through the inducement of cell apoptosis, sivH gene encodes an outer membrane protein associated with intestinal colonization [8,9].

Salmonella enterotoxin coded by *stn* gene, which play an important role in strain virulence, *Stn* gene designated as a virulence determinant in clinical strains of Salmonella enteric, its practical application for the inspection of the food and fecal samples[10]. Enterotoxin are resistance to high temperature, which belong to the broad family of pyrogenic toxin super antigens and have emetic activity. It is encoded pathogenicity islands, chromosomes, or plasmids[8]. Salmonella enterotoxin alters vascular permeability in the skin, increases cyclic AMP levels and exert substantial effects on water and electrolyte during intestinal infection. [11].

Material and methods

Sample collection:

A total 220 diarrhoeic stool samples were collected (most samples appeared mucoid and some was bloody), from the Rizkary, children's and Kirkuk hospitals in Kirkuk and Erbil city.

Identification culture mdia:

Pre-enrichment and Selective Enrichment:

The samples were added into a disposable plastic container containing 225ml of Peptone water(LAB, England). As a pre-enrichment medium, and incubated at 37°C for 16 hours. About 0.1 ml of the pre-enriched sample was transferred using a pipette into a tube containing 10 ml of tetrathionate broth (Himedia, India)(1:9) used for selective enrichment of samples, The samples were mixed well by shaking and incubated at 37°C for 18 hours[11].

Plating out and Identification:

A loop full of inoculums from broth cultures transsmitted were plated onto S.S agar (LAB, England), MacConkey's agar, XLD agar (Oxoid, England) and Kauffmann medium(LAB, England) , Triple sugar iron(TSI) (Oxoid, England) and selective 84369 Salmonella Chromogen agar media (Fluka, Switzerland) specifically designed for the differentiation of Salmonella colonies, isolates incubated at 37^0C for 24 hours aerobically in bacteriological incubator [12,13,14].

biochemical tests:

The pure suspected colonies were underwent biochemical tests such as fermentation of glucose achieved by stamped and streaking on Kligler Iron agar, urease reaction by cultured on Urea agar base, indole test by inoculated on peptone water,Voges prokauer and red methyl tests by inoculated onto Methyl red and Voges prokauer medium, H2S production hydrogen pyroxide reagent, In addition to the pure colony of *Samonella spp*. Examined by API20E (BioMerieux, France) which before explained by [13, 14, 15].

Susceptible test:

Isolates underwent to nine antibiotics sensitivity as showen in table (1), guidelines from the Clinical Laboratory Standards Institute [16,17,18], antimicrobial agents were tested, using the standard Kirby-Bauer disk diffusion method on Mueller-Hinton agar (LAB ,England).

Table 1 Elucidation antimicrobial disk in the study

Antimicrobial disk	Symbol	Disc potency	The company
Tetracycline	T	30 µg	Fluka , Switzerland
Augmentin(AC)	AC	20 /10 µg	Himedia ,India
trimethoprim-sulfamethoxazole	TMP	1.25 + 23.75 µg	Bioanalyse,France
Streptomycin	S	10µg	AL-razi
nalidixic acid	NA	30 µg	AL-razi
Cefotaxime	Cef	30 µg	Bioanalyse,France
Gentamycin	GM	10 µg	AL-razi
Ciprofloxacin	CIP	5 µg	AL-razi
Norfloxacin	NOR	10 µg	LAB, UK

DNA extraction: DNA was extracted by phenol-chloroform method according to [19]. DNA templates dissolved to determine DNA concentration at A 260 nm on ηg/ml, then resolved on agarose gel(Promega,US) as earlier described [19,20].

Detection *stn* gene: A specific PCR technique was accomplished by using one set of primer as shown in table(2) for finding *stn* gene that have a major role in most common symptoms of Salmonellosis. DNA magnification has done in a reaction volume of 25 µl, every reaction contained 1X PCR buffer, 1.5 µl dNTPs, 2.5µl DNA template 1.5 µl primer-F, 1.7primer - R, de ionized distilled water was added to make a final volume 25µl. each PCR products were

investigated in a 1.5 % agarose gel stained by ethidium bromide (10 mg/ml)(BDH,England), the thermocycler (Promega, US), PCR amplification program was 25 cycles of (94°C 1 min; 55°C 1 min, 72°C 1 min) to *stn* gene as previously described by[20] and then visualized using Gel Doc(Shimadzu, Japan). A DNA standard, 100 bp ladder (Promega, USA) have been maked use of as an indicator.

Table 2 Polymerase Chain Reaction Primers for stn Toxin Gene.

Oligonucleotide	Sequence	Expect product Size (bp)
stn – Reverse	CTT TGG TCG TAA AAT AAG GCG	260bp(Makino *etal*.,1999)[21]
Stn - Forward	TGC CCA AAG CAG AGA GAT TC	

Results

All samples were underwent for diagnoses using cultural and biochemical tests 72/220(32.7%) of samples gives: colourless in McConkey agar, red in Kauffmann medium, red colonies with black center on XLD, opaque on SS agar, choosen isolates were appeared negative to (indole, Voges-Proskauer, urease) and MR test positive, 41/72(57%) were *Salmonella typhimurum* and *Salmonella enteretedis* which produced alkaline red slants and acid yellow bottom with blackening and gas on TSI and red colonies (very good growth) on 84369 Salmonella Chromogen agar beside above tests, *Salmonella enteretedis* suspected colonies were further identified by biochemical tests (API 20E), which was presence in 28/41 (68. 3 %) [12, 13, 14].

In susceptibility test isolates appeared elevate resistance (100%, 80%, 72.3%) to Tetracycline ,Augmentin ,Trimethoprim in the order. At the same time streptomycin, nalidixic acid showed(62 %) each one and(50.2 %), (25 %), (16.7 %) for each of Gentamycin, Ciprofloxacin, Cefotaxime. While all isolates were Susceptible to Norfluxacin as showen in table 3 [17,18].

Table 3 explanation of antimicrobial sensitivity testing for all *S. pneumonia* isolates

Antimicrobial disk	Antibiotic sensitivity of *S. enteritidis*					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Tetracycline	15	62.5	9	37.5	-	-
Augmentin	3	12.5	16	66.7	5	20
trimethoprim-sulfamethoxazole	4	16.7	13	54.1	7	29.2
Streptomycin	5	20.8	10	41.7	9	37.5
nalidixic acid	8	33.3	7	29.2	9	37.5
Cefotaxime	-	-	4	16.7	20	83.3
Gentamycin	-	-	12	50	12	50
Ciprofloxacin	-	-	6	25	18	75
Norfloxacin	-	-	-	-	24	100

stn gene revealed the presence of *stn* gene in 24/28(85.7) , *Salmonella enteretedis* strains isolated from diarrhoeic patients by amplicon sizes 260 bp as shown in figure1, *stn* gene distributd to (7)29% in 60 above ages, (6)25% in ten to fifteen years, (5) 20% in each of one to twelve months and fifteen to twenty years and (1) 4.2% in one to five years, 9/28(32.1%) of cases in ages above sixty, while 7/28(25%)were at the age below one year and 6/28(21.4%) of cases were among ages between ten to fifteen years was recording positive to *Salmonella enteretedis* as shown in table 3 and figure 3.

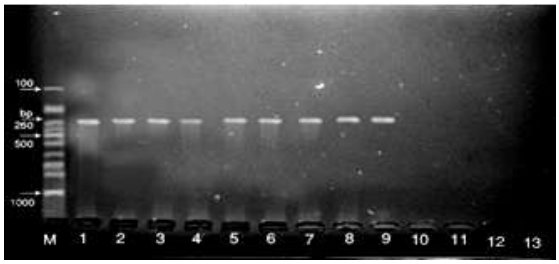

Figure (1) Gel *Agarose electrophoresis* showing an magnification of of *stn* gene (260 base pair) in *S. enteretedis* isolated from feces by using specific primer for *stn* gene on gel agarose 1.5%, Lane M: DNA ladder Marker(100 pb DNA ladder), lanes 1, 2, 3, 4, 5, 6,7,8,9: represent positive magnification process of *stn* in DNA of *S. enteretedis*, lanes 10, 11, 12, 13: represent negative control (did not appeared result in DNA of *Salmonella enteretedis*).

Discussion

Salmonellosis gastroenteritis owing to bacterial multiplication in intestinal submucosa and production of enterotoxins which elicits the inflammatory response of the host, salmonella tolerate gastric acidity some times disease occurs without bacteria reaches to stomach [22] ,endotoxins released from the dead salmonella cells at the decline phase when swallow salmonella booked by mucus formed in the esophagus that also causes poisonings[1, 4, 10]. Host cells in intestinal and gastrointestinal disorder (electrolyte problems) as aresponse to endotoxin effects that leads to diarrhoea [23]. Virulence factors responsible for pathogenicity in enteric bacteria are often plasmid encoded, transmission is fecal-oral and can occur through the ingestion of fecally contaminated food or water, or improperly cooked or

prepared food. Transmission may also occur via direct contact with an infected person, fomite, animal or an animal's environment [7,8]

S. enteritidis responsible for most diarrhoeic patients along with having *stn* gene, similar results have been recorded by other studies conducted around the world, [24,25] they found *stn gene* presence in all isolate of their study,they returne it to the global enhance of food-borne contaminations with antibiotic resistant microbes makes up a major public health problem, Antoine and his group found in 2008[24] both *Salmonella enteritidis* and *S*almonella typhimurium are the main serotype found in humans, plus they are also reported that the two above bacteria considered mostly recurrently isolated from bacteremia and from diarrheal diseases, Murugkar and his group[25] recorded in study conducted for the detection of the *stn* gene in 95 Salmonella isolates from five different serovars and four different sources has revealed that the gene was present in all the isolates including *Salmonella enteritidis*, and reports that the stn gene contained sequence unique to Salmonella strains, makes this gene a suitable PCR target for detection of Salmonella strains in field samples, Shrivastav and his group[26] reported presence of *stn* in19/24 (79.2%) isolate. In another study achieved by Purkayastha with his group[27] isolated Salmonella from various sources such as animals, birds and human they among the 30 isolates recovered, the serovar Salmonella enteritidis was found to be the most frequent serovar (53.33%), they also examined some virulence genes. All the 30 Salmonella isolates showed presence of *invA* and *stn* genes suggesting the possibility of using these genes for identification of Salmonella. These findings are in agreement with the findings from the present study which show the gene *stn* is present in 24/28(85.7%) Salmonella *enteritidis*, these widely distributed of *stn* gene among the Salmonella supporte earlier reports and leads to suggesting that *stn* has significant role in the pathogenesis of this bacteria and understand of *stn* mechanisms and founding of genetic profiles for these microbes can be used to determine patterns of virulence, These may help develop tools to expect the ability of pathogenesis of *Salmonella enteritidis* it can be a viable target gene to explore the possibility of direct detection of Salmonella from samples from biological sources [28].

Table 4 Shows prevalence of *S.enteretedis* and *stn* among different ages groups in human.

Age of patients		No. of tested samples	Positive (*S.enteretedis*)		Positive *stn*	
			No.	%	No.	%
Month(1-12)		44	7	25	5	20.8
Years	1-5	20	1	3.6	1	4.2
	5-10	13	–	–		
	10-15	30	6	21.4	6	25
	15-20	25	5	17.9	5	20.8
	20-25	18	–	–	-	-
	25-30	6	–	–	-	-
	30-35	8	–	–	-	-
	35-40	12	–	–	-	-
	40-60	17	–	–	-	-
	60-above	27	9	32.1	7	29.2
Total		**220**	**28**	**12.7**	**24**	**85.7**

The finding from current study show prevalence of salmonella in old, infants(10-12)month, adolescence and in (15-20) years in the order. Elderly, pregnant, infants, children, and people between (15-20) years at danger for severe complications as a result of Salmonella food poisoning [29, 30].

The Infection exposure among these age groups may returns that they are less protective. The difference in the prevalence of Salmonella among age group may be due to variation in the response to infection with a Salmonella species challenge dose among age groups and the immunological status of the person which previous exposure to infection and exposure to stressors, particularly in older group[31]. It is also some precipitating factors such as concurrent disease, weakness and inability to give attention to personal hygiene[32]. While for adolescent the lack of attention to healthy foods or eat more prepared foods(tins)may cause the disease in old, infants and adolescent than another ages. In Africa, NTS has consistently been reported as a leading cause of bacteremia among immunocompromised people, infants and newborns[28]which compatible with this study, Presnt study compatible with Zhaoming etal [33] that reported epidemiologic characteristics of NTS infections in Guangdong Province from 2009 to 2012, the study showed that 73% children aged under five years were the group most affected by NTS. While in previous study by Olsen *etal* [34] in 1987 to1997 recorded the NTS isolation rate was highest in patients aged under one year Future study must investigations about risk factors to determine

why children especially infants and old have become the majority of infections for disease control and prevention.

This study compatible with zoonotic agents and food borne which provided in 2014, The fact that the elderly, young children and those with weakened immune systems are most at risk for developing salmonellosis and suffering severe reactions futher more Infants and elderly are the most exposed at risk to poisoned, which with no trouble reached by Swallowing a little number of bacteria, while in healthy adults must be ingested in large numbers to cause sickness[32,35]. The most ingest bacteria damaged by gastric acidity, Salmonella has ability to tolerant and survived under acidic conditions as a result of Salmonella possess inducible acid tolerance system which is important to the virulence of the organism[22].

Increasing antibiotic resistance was noticed in this study, mainly to Tetracycline, Augmentin, Trimethoprimand Streptomycin, Nalidixic acid, NTS isolates in study accomplished in Malaysia displayed elevated resistance to Tetracycline, Sulfonamides and Streptomycin in rates (70%), (57%), (53%) respectively but minor rates(28%) to Nalidicix acid .Multidrug resistance in China is seemed to be complicated, additional displation found out the appearance resistant NTS isolates are required to discontinue this hazardous situation [36].

To turn off the outbreak needs to control all stages of the food chain, from agricultural production till handing out, in addition to preparation of foods that was made in junk foods or at home according to International Network of Food Safety Authorities (INFOSAN) Recommendations.

Third generation of Cephalosporins(Cefotaxime) and Fluoroquinolones (Ciprofloxacin ,Norfloxacin) the first choice for cure of salmonellosis [37], [38]. An increase in the number multi drug resistance strains may leads to treatment failure. Cephalosporins is successful drug suggested for children, Nalidixic acid susceptibility testing has been recommended by [16] before using Ciprofloxacin for treatment, isolates that resistant to Nalidixic acid are probably resistant to Ciprofloxacin [38, 39]. Resistance to older antibioticl agents Tetracycline Amoxicillin-Clavulanic acid, Trimethoprim-Sulfamethoxazole and Streptomycin has been present for several years; these must not be considered first-line empiric agents in treatments[39].

In moderate cases of salmonellosis patients does not needs to give them the cure especially among adults, in this cases they were get recover through several days, but they may be required fluids in dehydration situations.While elderly and infants necessary to take therapy to avoid infection broaden[32].

In conclusion Prevalence multidrug resistance and *stn* gene among *S. enteritidis* isolated from diarrhoeic patients and most of the cases were among ages above sixty and less than one year.

So this study suggest that future studies should look more into NTS as causes of diarrhea infections. Sources and ways of transmission of antimicrobial resistance and multidrug resistance among NTS should be continuously monitored and epidemiological surveillance programs.

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الكشف عن جين *stn* في بعض عزلات السالمونيلا اللاتيفوئيدية والمعزولة من مرضى الاسهال

شاميران محمود توفيق

قسم التربة والمياه ، كلية الزراعة ، جامعة صلاح الدين ، اربيل ، العراق

الملخص

هدفت الدراسة الحالية لمعرفة نسبة الاصابة ببكتريا السالمونيلا اللاتيفوئيدية *Salmonella* enteritidis، جمعت العينات من المرضى المصابين بالاسهال في مستشفيات رزكاري في اربيل و كركوك للفترة من نيسان2017 الى ايلول 2017وللفئات العمرية من شهر ولحد عمر فوق60 سنة، وبينت نتائج التشخيص واعتمادا على الفحوصات المورفولوجية، الزرعية، الاختبارات الكيموحياتية والتشخيص باستخدام عدة Api 20-E 32.7% (72)عينة اعطت نتيجة موجبة لبكتريا *Salmonella sp* ، 57% (41)عزلة شخصت عائدة لبكتريا السالمونيلا اللاتيفوئيدية و68%(28) عزلة كانت عائدة لبكتريا *S.enteritidis*،كما بينت نتائج التحري عن جين الضراوة *stn* ان 85.7% (24) كانت نسبة انتشار الجين بين عزلات *S.enteritidis* ،واظهرت العزلات 100% حساسية تجاه مضاد نورفلوكساسين و 16.7% تجاه سبروفلوكساسين، بينما ابدت 70% من العزلات تعددية مقاومة تجاه، اشارت نتائج هذه الدراسةان 32.1%نسبة الاصابة بين اعمار فوق الستين و25% في اعمار دون السنة.