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

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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 induction 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 mediu:
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°C 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 Salmonella spp. Examined by API20E (BioMerieux, France) which before explained by [13, 14, 15].

Susceptible test: Isolates underwent to nine antibiotics sensitivity as shown 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-      | TMP    | 1.25 + 23.75 µg | Bioanalyse,France |
| sulfamethoxazole   |        |              |             |
| Streptomycin       | S      | 10 µg        | 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 ng/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, 1.7 primer - R, de ionized distilled water was added to make a final volume 25µl. each PCR products were
investigated in a 1.5 % agar 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 made use of as an indicator.

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

Table 2 Polymerase Chain Reaction Primers for stn Toxin Gene.

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 typhimurium and Salmonella enteretidis 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 enteritidis 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, Gentamycin, Ciprofloxacin, Cefoxamine. While all isolates were Susceptible to Norfloxacin as shown in table 3 [17,18].

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

| Antimicrobial disk | 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 |
| Cefoxamine         | -   | -   | 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 enteritidis strains isolated from diarrhoeic patients by amplicon sizes 260 bp as shown in figure 1, 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 enteritidis 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. enteretidis 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. enteritidis, lanes 10, 11, 12, 13: represent negative control (did not appeared result in DNA of Salmonella enteritidis).

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 diarrhea[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

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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 return 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 Salmonella 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 diarrhoeal 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* in 19/24 (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 finding 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.enteritidis* and *stn* among different ages groups in human.**

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

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, Present 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 to 1997 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 further 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, Trimethoprim and 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 Nalidixic 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

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