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RESEARCH ARTICLE

PREVALENCE OF MULTI-DRUG RESISTANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM-ASSOCIATED DIARRHEA IN A SAMPLE OF IRAQI HOSPITALIZED CHILDREN

[1]*Safa K. Ibraheem, [1]Abdulwahid B. Alshaibani, [2]Ali H. Ad'hiah

[1]Biotechnology Department, College of Science, Al-Nahrain University, Baghdad, Iraq
[2]Tropical-Biological Research Unit, College of Science, University of Baghdad, Baghdad, Iraq

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ABSTRACT

The investigation aimed to determine the prevalence of *Salmonella enterica* serovar Typhimurium (S. Typhimurium) in hospitalized diarrheal children, together with its multi-drug resistance (MDR) status. A total sample of 128 patients (76 males and 52 females) was enrolled in the study. They were hospitalized children due to diarrhea and fever, and admitted to the Central Pediatric Hospital in Baghdad during the period April - September, 2012. Their ages had a range of six days and up to five years. *S.* Typhimurium was isolated and identified from nine stool samples (7.03%), which were belong to nine children. All isolates were resistant to nalidixic acid, with the exception of isolate 1.11, which showed intermediate sensitivity. Only isolate B was resistant to three antibiotics, which were ampicillin, amoxicillin/clavulanic acid and nalidixic acid. These results demonstrate that S. Typhimurium is an important causative pathogen of diarrhea in children, especially if we consider its MDR.

INTRODUCTION

Salmonella is one of the principal causative agents of poisoning and food-borne disease in the world, and enteric infections caused by this genus are one of the major causes of morbidity and mortality in infants in developing countries. It can cause a wide variety of diseases; from mild gastroenteritis to typhoid fever, and nature and severity of the infection are dependent on many factors; including the serovar involved, the virulence of the strain, the infective dose, the species, the age and immune status of the host (Gordon, 2011; Kaiser and Hardt, 2011). Accordingly, it has been stated that salmonellosis is an important global public health problem caused by the *Salmonella* spp., which are the main cause of typhoid fever in humans, and responsible for a serious health problem in developing countries. Twenty two million new cases of typhoid fever are registered annually worldwide despite various vaccination programs (Gunn *et al*., 2014), and in children, *Salmonella* infection is an important health risk, and an estimated 17 million cases of typhoid fever (enteric fever) with approximately 600,000 deaths worldwide each year has been reported, in which *S. enterica* serovar Typhimurium (*S.* Typhimurium) is a major cause of food-borne illness, causing intestinal inflammation and diarrhea (Waddington *et al*., 2014).

Corresponding author: Safa K. Ibraheem
Biotechnology Department, College of Science, Al-Nahrain University, Baghdad, Iraq

A major concern of *S.* Typhimurium for physicians in developing countries is their antibiotic resistance, and multidrug-resistant (MRD) strains continue to be a worldwide health challenge (Tadesse, 2014). Accordingly, the present investigation came to shed light on the prevalence of this pathogen in a sample of hospitalized children due to diarrhea. Equally important, the antibiotic resistance of the pathogen was also investigated.

MATERIALS AND METHODS

Patients and Sample Collection

One hundred and twenty eight patients (76 males and 52 females) were enrolled in the study. They were hospitalized children due to diarrhea and fever, and admitted to the Central Pediatric Hospital in Baghdad during the period April - September, 2012. Their ages had a range of six days and up to five years. A stool sample was taken from each pediatric patient upon admission to the hospital and before taking any medication using a disposable swab applicator, which was transferred to a test tube containing 10 ml sterilized peptone water. The tube was transferred to the laboratory (2-3 hours), and then the sample was incubated at 37°C with a total time of approximately 24 hours.

Bacterial Isolation and Identification

After incubation, 5 ml of cultured stool sample were transferred to 250ml flask containing 50 ml tetra-thionate broth (an enrichment medium for the selection of *Salmonella*), and incubated at 37°C for 24 hours. After incubation a loopful from each flask was streaked on SS agar (selective medium) plates, and incubated at 37°C for 24 hours. The suspected colonies were examined by Gram stain and then subjected to catalase, indole, methyl red, Vogas-Proskauer, citrate utilization, Kligler iron and glucose fermentation tests (Atlas *et al*., 1995; Collee *et al.*, 1996). The *Salmonella* was further identified by APi 20E system, while *S*. Typhimurium was identified by serotyping, in which a panel of antisera (anti-Omni, anti-OMA, anti-O serum, anti-HMA and anti-H sera; BioRad Company, USA) were employed.

Antibiotic Susceptibility Test

Susceptibility of *S.* Typhimurium isolate to different antibiotics was studied by the standard disk diffusion test of National Committee for Clinical Laboratory Standards (NCCLS) of 2002. Five milliliters of sterile brain heart infusion broth were inoculated with 0.1 ml of the fresh culture of *S.* Typhimurium isolate and incubated at 37°C for 4 hours in shaking incubator (100 rpm). Then, ten serial dilutions were prepared, and 0.1 ml of the fourth dilution (10^{-4}) was spread on brain heart infusion agar plate. The inoculated plates were placed at room temperature for 30 minutes (to allow absorption of excess moisture), before antibiotic discs were placed on the inoculated plates (5 discs / plate), and incubated at 37°C fo18 hours. After incubation, diameters of the inhibition zones were measured and compared with that of NCCLS.

RESULTS AND DISCUSSION

Prevalence of *S.* Typhimurium

Out of 128 stool samples, *S*. Typhimurium was isolated and identified from nine samples only (7.03%), which were belong to nine children; their ages ranged from six days to five years. All children had severe diarrhea that was associated with fever. In this context, t has been well-documented that enteric infection with *Salmonella* spp. is an important cause of children diarrheal disease worldwide, but the frequency of children with the infection shows variations between studies. A study from Bolivia of 133 consecutive children less than 5 years old presenting with bloody diarrhea revealed bacterial etiology in 41%, and out of this percentage, *Salmonella* spp. accounted for 4% (Townes *et al*., 1997). However, in a further study from Vietnam, no *Salmonella* infection was reported in 587 children with diarrhea under the age 24 months, although other potential pathogens were identified in 67.3% of children with diarrhea, including Gram negative bacteria (Vu Nguyen *et al*., 2006). In a more recent study, *Salmonella* spp. infection was reported in 6.2% of 260 diarrheal children from Ethiopa (Beyene and Tasew, 2014). These findings together with present study findings confirm that globally, intestinal *Salmonella* spp. remain major contributors to acute enteric infections.

Isolation and Identification

The isolation of *S*. Typhimurium was performed by cultivating the stool samples in flasks containing 50 ml tetra-thionate broth medium, which was used as a selective enrichment for the cultivation of *Salmonella* spp that may be present in small numbers and compete with intestinal flora. Selectivity is accomplished by the combination of sodium thiosulfate and tetra-thionate, which suppresses common intestinal organisms (Eckner *et al*., 1994) For pure cultures, 100µl aliquots from tetra-thionate broth cultures were transferred and spread onto the surface of *Salmonella-Shigella* agar plates (SS agar), which is highly selective and differential medium formulated to inhibit the growth of most coliform organisms and permit the growth of *Salmonella* and *Shigella* species from environmental and clinical specimens (Murray *et al*., 2007). The high bile salt concentration and sodium citrate inhibit all Gram positive bacteria and many Gram-negative organisms including coliforms. Lactose is the sole carbohydrate and neutral red is the indicator for acid detection. Sodium thiosulfate is a source of sulfur and the production of H_2S is indicated by black precipitate formed with ferric citrate (Atlas *et al*., 1995). The growth of *Salmonella* spp. in SS agar showed colorless colonies with black centers owing to H_2S production, while *Shigella* spp. does not blacken (Wells and Butterfield, 1997). As a result, nine isolates, which showed black colonies in SS agar, were suspected as *Salmonella* spp. and subjected for further identifications.

The suspected isolates were first identified depending on their Gram stain and microscopical characteristics. *Salmonella* isolates were found to be Gram negative, rod or bacilli and non-spore forming. Morphological characteristics of the suspected isolates were done depending on the colonial shape and form when re-cultured on the surface of MacConkey agar and SS agar. The colonies were pale yellow or nearly colorless, 1-3 mm in diameter and non-lactose fermented. In addition, the colonies in SS agar appeared to be circular, colorless, 1-2 mm in diameter with gas bubbles and black spots due to the production of H_2S. Such characteristics were applied to only nine isolates that were suspected to be candidate for the biochemical tests.

Biochemical tests were achieved on the suspected nine isolates that showed growth and morphological characteristics, which were closely related to *S*. Typhimurium. For this purpose eight biochemical tests were carried out. Results showed that the nine isolates gave negative reactions for indole, urease and Voges-Proskaur tests, but they gave positive reactions for citrate utilization, catalase, methyl red and glucose fermentation tests. In triple sugar iron test, both isolates were H_2S and gas producers (Holt *et al*., 1994). Enterobacteriaceae members are Gram-negative bacteria and can grow in the intestinal tract of human and animals (Prescott *et al*., 1996). Many conventionl biochemical tests have been used to distinguish between Enterobacteriaceae family. *Salmonella* is a pathogenic enterobacter, which can be identified from coliforms in many tests; for instance, H_2S production, blackening and non- lactose fermentation (Woo *et al*., 2001).

Within the genus *Salmonella*, species can be characterized by carrying out more biochemical tests (i.e. fermentation of different sugars), serological and genetic examinations; however species of *Salmonella* also differ from each other in the conventional biochemical tests; for instance, *S.* Typhimurium can utilize citrate but not *S.* Typhi or *S.* Paratyphi A (Tindall *et al*., 2005). A further identification of the nine *S.* Typhimurium isolates was achieved by using Api system (Api 20E). Results of these isolates were able to give positive results for ornithine decarboxylase, citrate utilization, H_2S production, gelatin liquefaction, and they were glucose, arabinose, sorbitol, rhamnose, melibiose, inositol and manitol fermentors. While, they gave negative reactions for β-galactosidase, arginine dihydrolase, lysine decarboxylase, urease, tryptophane deaminase, indole , Vogas-proskauer, amygdaline fermentation and sucrose fermentation. The results mentioned above were in agreement with those described by Holt *et al*. (1994); indicating that the nine isolates were belong to the genus *Salmonella*., and by applying serotyping, they were confirmed as *S*. Typhimurium.

Antibiotic Susceptibility

Disc diffusion method was used for testing the susceptibility of *S.* Typhimurium to six antibiotics (ampicillin, amoxicillin/ clavulanic acid, nalidixic acid, ciprofloxacin, trimethoprim/ sulfametho-xazole and azithromycin. The nine tested isolates showed different susceptibilities to the tested antibiotics, and most of them were sensitive, others showed intermediate sensitivity, and resistant isolates were also observed. All isolates were resistant to nalidixic acid, with the exception of isolate 1.11, which showed intermediate sensitivity. Only isolate B was resistant to three antibiotics, which were ampicillin, amoxicillin/clavulanic acid and nalidixic acid; therefore this isolate was considered as multi-drug resistance (MDR) isolate (Table 1).

Table 1. Susceptibility of *Salmonella* Typhimurium to six antibiotics.

Isolate Symbol	AMP	AMC	NAL	CIP	SXT	AZ
1.1	S	S	R	S	I	S
1.2	S	S	R	S	S	S
1.3	S	S	R	S	S	I
1.5	S	S	R	S	S	S
1.6	I	S	R	S	S	S
1.7	S	S	R	S	S	S
1.11	S	S	I	S	S	S
A	S	S	R	S	S	S
B	R	R	R	S	S	S

AMP: Ampicillin, AMC: Amoxicillin/clavulanic acid, NAL: Nalidixic acid, CIP: Ciprofloxacin, SXT: Trimethoprim/sulfamethoxazole, AZ: Azithromycin, I: Intermediate sensitivity, S: Sensitive, R: Resistant

As shown in the table, *S.* Typhimurium B isolate was sensitive to three antibiotics (ciprofloxacin, trimethoprim/ sulfametho-xazole and azithromycin), but it resisted ampicillin, amoxicillin/clavulanic acid and nalidixic acid. In agreement with such presentation, *S.* Typhimurium has been documented to be frequently associated with MDR (Soto *et al*., 2003; Biendo *et al*., 2005), in part due to the worldwide emergence of *S.* Typhimurium definitive phage type (DT) 104, which contains the chromosomal *Salmonella* genomic island type I (SGI-1).

SGI-1 harbors genes that confer the ACSSuT phenotype (i.e., resistance to ampicillin, chloram-phenicol, streptomycin, sulfonamides, and tetracycline) (Mulvey *et al*., 2006). Non-typhoidal *Salmonella* infections generally result in mild-to-moderate self-limiting gastroenteritis, and antimicrobial treatment is only required in severe cases occurring in vulnerable patient groups or to combat invasive infections. However, due to the increasing resistance of this bacterium to the conventional anti-microbial agents used in the treatment of salmonellosis, amoxicillin/clavulanate, third-generation cephalosporins, and fluoroquinolones have become further treatment options. Resistance to β-lactams in *S. enterica* is mainly due to the production of acquired β-lactamases (Michael *et al*., 2006). Among these, TEM-1, PSE-1, and OXA-1 have been described as the enzymes most frequently related to ampicillin and amoxicillin/ clavulanate resistance (Güerri *et al*., 2004; Biendo *et al*., 2005). The resistance of *Salmonella* to third-generation cephalosporins is primarily mediated by the production of extended spectrum β-lactamases (ESBL) of the TEM, SHV, and CTX-M types, which are associated with different mobile genetic elements (Michael *et al*., 2006). ESBL have been described not only in clinical *Salmonella* isolates but also in isolates from animals and food (Coque *et al*., 2008).

Mobile genetic elements such as plasmids and transposons, possibly containing integrons, are able to disseminate anti-microbial resistance by horizontal transfer in Enterobacteriaceae. Integrons are genetic elements that capture and incorporate gene cassettes by using a site-specific recombination mechanism (Cambray *et al*., 2010). In recent years, resistance to amoxicillin/ clavulanate among *S. enterica* isolated from different hospitalized cases (including the present patients) has become increasingly widespread, accompanied by the emergence of ESBL-producing isolates, detected in human samples. Consequently, there are fewer therapeutic options for the treatment of *S. enterica* infections, placing these patients at greater risk of serious morbidity and even death. This resistance may be related to resistance gens possessed by the bacteria (Wain *et al*., 2013). Levings *et al*. (2005) also found that this pathogenic bacterium was resistant to gentamycin and streptomycin, and during the period 1999 - 2008, Matheson *et al*., (2009) mentioned that, *Salmonella* strains increased their resistance to chloramphenicol; a matter which may reflects that such new strain may carry mobile genetic elements conferring resistance to multiple antibiotics. High sensitivity of *S.* Typhimurium for ciprofloxacin and its resistance to ampicillin were also recorded by Mitra *et al*. (2009). Another study performed by Kasimoglu Dogru *et al*. (2010) found that *Salmonella spp*. isolated from chicken carcasses possessed MDR toward tetracycline, cephalothin, and streptomycin. In conclusion, it is possible to consider that S. Typhimurium as an important causative pathogen of diarrhea in children, and special attention must be paid to its MDR.

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