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Detection of Multi-drug Resistant Non-typhoid *Salmonella* Isolates in Cases of Gastroenteritis in Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The current study aimed to determine the prevalence and multidrug resistance of non-typhoid *Salmonella* in patients with gastroenteritis at Minia, Egypt. The presence of *tet*B gene and *cat* gene was determined by PCR to have a clue of the resistance mechanisms to tetracycline and chloramphenicol as available and cheap drugs in treatment of non-typhoid *Salmonella* infections in developing countries.
Methodology: Five hundred stool samples were collected from patients with gastroenteritis, attending Minia Fever Hospital, Egypt, in the period from August 2011 to January 2014, all the participants showed negative- Widal test that was necessary to be included in the study. The stool samples were examined by standard microbiological, biochemical, *inv*A gene amplification by PCR

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and serological tests to isolate non-typhoid *Salmonella.* The antimicrobial susceptibility was tested by the disc diffusion method using a panel of 11 discs of different antimicrobial groups, the Production of extended-spectrum β-lactamase was detected using the double disk synergy test. The presence of *tet*B gene and *cat* gene was determined by PCR in tetracycline and chloramphenicol resistant isolates respectively.

Results: Of 500 samples, 4.4% (22/500) were non-typhoid *Salmonella.* Most of isolates were resistant to; ampicillin 86.4% (19/22) then tetracycline and trimethoprim-sulphamethoxazole 77.3% each (17/22), 27.3% of isolates (6/22) were resistance to chloramphenicol. However low percentage of isolates were resistant to quinolones and most of isolates (95.5%) were sensitive to amikacin and ciprofloxacin. 18% (4/22) of isolates were ESBL producers and 81.8% (18/22) were multiple drug resistant (MDR). *tet*B and *cat* genes were detected in 64.7% (11/17) and 50% (3/6) of isolates resistant to tetracycline and chloramphenicol respectively.

Conclusion: This study revealed high prevalence of MDR non-typhoid *Salmonella* isolates that represents a serious health problem in the region under study. Quinolones remain the treatment of choice, while amikacin can be used in children. Continuous search for the mechanisms of resistance by molecular studies is important for effective management of NTS infections in Egypt.

Keywords: Cat gene; non-typhoid Salmonella; multiple drug resistant; tetB gene.

1. INTRODUCTION

The genus *Salmonella* are facultative anaerobes, non-spore forming and Gram negative motile bacillus belonging to the family *Enterobacteracae*. The virulence of *Salmonella* is related to chromosomal and plasmidiail factors, the chromosomally located invasion gene, *inv*A gene is essential for invasion of the mammalian epithelial cells [1,2]. There are nearly 2700 *Salmonella* serotypes, based on somatic, flagellar and capsular antigens [3], and only those contain *inv*A genes can invade host cells [3,4]. The *inv*A gene has unique sequences to *Salmonella*, so the detection of the presence or absence of the gene by PCR is considered as a gold standard of *Salmonella* infection identification [4].

The true incidence of non-typhoid *salmonella* (NTS) infections is not known, but there are about 93.8 million episodes of NTS infections and 155,000 deaths worldwide every year [5].*Salmonella* Typhi and *Salmonella* Paratyphiinfect humans only but NTS can affect both animal and humans, poultry and eggs are the main source of NTS [6,7].

In Egypt, NTS infections in poultry have increased in the last years. For instance, some reports showed that the incidence of *Salmonella enterica* seovar Typhimurium and *S. enteric* serovar Enteritidis in eggs were 16.7% and 13.3% respectively [8]. NTS organisms are the commonest cause of bacterial enteritis in children and bloodstream infections mainly in several African countries [9]. In NTS gastroenteritis, antibiotics are recommended for patients with severe illness or invasive disease [10].Antibiotic resistance in *Salmonella* was firstly reported in the 1970s as chloramphenicol resistance but in last years multidrug resistance (MDR) was commonly observed [11]. The resistance to some antibiotics, such as β-lactam, tetracycline, or chloramphenicol is increasing, this is a big challenge for the treatment of children because fluoroquinolons should not be used in this age group [12]. *Salmonella* resistance to chloramphenicol is caused by two mechanisms: the plasmid-located enzymes called chloramphenicol acetyltransferases (*cat*) or nonenzymatic chloramphenicol resistance gene [13]. Regarding tetracycline, the majority of *tet* genes that confer resistance to this antibiotic belong to classes A, B, C, D and G [14]. The *tet*B gene is present on the chromosomes and transferable plasmids of many *Salmonella* serotypes, mostly found in MDR *Salmonella* isolates. This gene is being considered as an important marker in determining potentially serious *Salmonella* infections [13,15]. The current study aimed to determine the prevalence and multidrug resistance of non-typhoid *Salmonella* in patients with gastroenteritis at Minia Fever Hospital, Egypt. The presence of *invA* gene, *tet*B gene and *cat* gene was also determined by PCR since these genes can give a clue of the resistance mechanism of tetracycline and chloramphenicol in resistant isolates and be used for identification of potentialserious NTS infections.

2. MATERIALS AND METHODS

Participants Five hundred patients attending Minia Fever Hospital with nausea, vomiting, fever, abdominal pain and diarrhea (gastroenteritis), participated in the study in the period from August 2011 to January 2014. The study was carried out in the department of Microbiology and Immunology, Faculty of Medicine, Minia University, Egypt. All participants showed negative-Widal test that is routinely performed in the fever hospital lab. The study proposal was approved by the Council of Faculty of Medicine and the Fever hospital. Each patient consented prior to participation.

2.1 Isolation and Identification of Salmonella

A total of 500 stool samples were collected and examined by standard microbiological and biochemical tests. 0.5 ml of stool suspension in saline solution was inoculated into pre-enrichment Broth (Selenite F) (Himedia, INDIA) for 18 h, then subcultured on xylose-lysine-deoxycholate (XLD) agar (OXOID, UK), MacConkey agar and Salmonella-Shigella agar (SS agar) plates (OXOID, UK) for 24 h at 37°C. Suspected colonies were examined biochemically using TSI, urea agar, simmon citrate agar, indole medium, SIM medium, MRVP test and lysine decarboxylation (MERCK Company Supplier, Germany) [16].

2.2 Serological Identification of NTS

The isolates were subjected to serological identification of NTS to exclude S. Typhi and S. Paratyphi by slide agglutination test using polyvalent O and H antisera (Meurex Biotech Ltd, UK) according to the Kauffmann-White scheme [17]. Isolates were kept frozen at –20°C in brain heart infusion broth containing 15% glycerol for further testing.

2.3 Antimicrobial Susceptibility Testing

The antibiotic sensitivity was performed by disc diffusion method [18] using the following antimicrobial discs: tetracycline (30 µg), chloramphenicol (30 µg), sulphamethoxazole-trimethoprim (25 µg), ampicillin (10 µg), streptomycin (10 µg), nalidixic acid (30 µg), ofloxacin (10µg), ciprofloxacin (² µg), amikacin (30 µg), ceftazidime (30 µg), and cefotaxime (30 µg) (all from Bioanalyse, Turkey). Muller Hinton Agar plates were inoculated with 0.5 Mc Farland standard suspension of the isolates, antibiotic disks were placed and incubated for 24 h at 37°C. Zone diameters were measured and interpreted according to the guidelines of the Clinical Laboratory Standard Institute [19]. Resistant isolates to at least one member of three different antimicrobial groups are considered as MDR [20,21].

2.4 Extended-Spectrum β-lactamase (ESBL) Detection

The Production of ESBL was detected using the double disk synergy test (DDST) for detecting the mechanism of reduced susceptibility to third generation cephalosporins. The DDST was performed by using; cefotaxime (30 µg),ceftazidime (30 µg), and ampicillin (10 µg) each at a distance of 30 mm away (centre to centre) from a disk containing augmentin (amoxicillin 20 µg and clavulanic acid 10 µg). By comparing the inhibition zone of disks, when zones were enlarged more than 5 mm around the disk containing clavulanic acid the isolate were considered as ESBL positive [22].

2.5 DNA Extraction and PCR Amplification

DNA was extracted from the suspected isolates (22/500) using genomic BYF DNA extraction Mini kit (Intron Biotechnology, Korea) according to the manufacturer's instructions. The *inv*A gene was amplified by PCR in the biochemically confirmed isolates (N=22) and four different isolates (two *Escherichia coli* and two *Shigella dysenteriae*) that were isolated from our stock collection were used as a control. The primer set used (Eurofins, Germany) described in Table 1. PCR was performed in a 25 µL reaction mixture containing 1 µL of template DNA (approximately 100 ng/µL), 12.5 µL of PCR master mix (2X DreamTaq® Green Master Mix, Fermentas, Lithuania), 1 µL(10 pmol) of each primer and 9.5 µL of nuclease free water. The *inv*A amplification was carried out in a thermal cycler (Techine TC 512, UK) as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of 1 min of denaturation at 94°C, 30 seconds of annealing at 60°C and 1 min of extension at 72°C and a final extension step at 72°C for 10 min.

The tetracycline resistance-related gene (tetB) and chloramphenicol acetyltransferase gene (cat) were amplified by PCR using the primers sets (Eurofins, Germany) described in Table 1. PCR reactions were performed using the same

conditions for invA gene except the annealing temperature, which was 56°C for *tet*B and 50°C for cat. PCR products were resolved on 1.5% agarose gel and visualized under a UV transilluminator (Biometra, Germany).

2.6 Statistical Analysis

Statistical analysis were performed by SPSS version 11.0. Levels of significance determined by *p* value using independent *t*-test were interpreted as follows: *p* values ≤0.001, ≤0.05 and ≥0.05 indicated high significance, significance and insignificance, respectively.

3. RESULTS AND DISCUSSION

3.1 Characteristics of the Study Population and Identification of NTS

Out of 500 samples, 4.4% of the isolates (22/500) were identified as NTS by standard microbiological, biochemical and serological methods. For all the biochemically confirmed isolates as *Salmonella* (N=22), it was performed the amplification of *inv*A gene by PCR (Fig.1). The gene was detected in all of them, which agrees with other studies that found *inv*A gene in all isolates diagnosed as *Salmonella* by microbiological methods [26-28]. Similar frequencies of NTS isolation were reported by other researchers 3.73% [29] and 8% [30]. However a high frequency of isolation was reported in Yemen (16.4%) [31], and low frequency (0.9%) was also reported in Saudi Arabia [32]. The low rate of isolation of NTS was expected, and it may be due to a prior treatment of patients, since they were hospitalized. Additionally in poorly countries patients suffering from mild to moderate diarrhea do not seek treatment.

The difference between the mean age of the participants and the age of patients positive to NTS was statistically insignificant (p=0.3) as well as the gender (p=0.5), or the residence predilection (p=0.09) among the patients with NTS, which agrees with previous studies [33].

3.2 Antimicrobial Resistance of NTS Isolates

Antimicrobial resistant NTS are associated with treatment failure and complicated diseases [34]. There is an increase in antibiotic resistance rates among NTS worldwide, in spite of the significant geographical and serotype variability [35,36]. Inour study antimicrobial susceptibility of 22 NTS isolates was performed using 11 antimicrobial agents. As shown in Table 2 and Table 3, isolates display high resistance to ampicillin86.4% (19/22) and 77.3% (17/22) to tetracycline and trimethoprim-sulphamethoxazole. Subsequently, streptomycin 31.8% (7/22) and chloramphenicol 27.3% (6/22) were the antibiotics that showed highest resistance rates. These resistance rates are consistent with reports from other middle to low income countries, which reported also high rates of ampicillin, and trimethoprim-sulfamethoxazole resistance [9].

Cephalosporins are commonly used to treat complicated *Salmonella* infections. However, their efficacy is being doubted by the emergence of extended-spectrum β-lactamases (ESBLs) and plasmid mediated cephalosporinases. Our study showed that 31.8% (7/22) and 50% (11/22) of isolates were resistant to cefotaximeand ceftazedeme, respectively. VO et al 2010 have reported that 100% (N=297) of NTS isolates were susceptible to both cefotaxime and ceftazedeme [37].

Most of isolates were sensitive to ciprofloxacin (quinolone) and amikacin 95.5% (21/22) which agrees with previous studies [31,32], therefore amikacinseems to be a good choice mainly in children's treatment among the patients understudy. However, quinolones such as ciprofloxacin could remain the treatment of choice for the majority of the cases, although we have found that 27.5% (6/22) of isolates were resistant to ofloxacin and 22.5% (5/22) were resistant to nalidixic acid.

Eighteen isolates (81.8%) were resistant to at least one member of three different antimicrobial groups (MDR). Other studies have reported 100% (N= 150, 167) of isolates were MDR [38,39] but Firoozeh et al. [40] have reported that 74.1% (N=43) of NTS isolates were MDR.

ESBL production in the current study was detected phenotypically in 18% (4/22) of isolates which is relatively high comparing with other studies [33,41].

Table 1. Primers used in the amplification of *tetB*, *cat* and *invA* genes

Gene	Primer sequence	Ampliconsize	Reference
*inv*A	F'GTGAAATTATCGCCACGTTCGGGCAA-3' R 5'- TCATCGCACCGTCAAAGGAACC-3	284bp	[23]
tetB	F 5' GAGACG CAA TCG AAT TCG 3' R 3'TTTAGTGGCTATTCTTCCTGCC5'	227bp	[24]
cat	F: 5' GGT GAT ATG GGA TAG TGT T 3' R:5' CCATCA CAT ACT GCA TGATG 3'	349bp	[25]

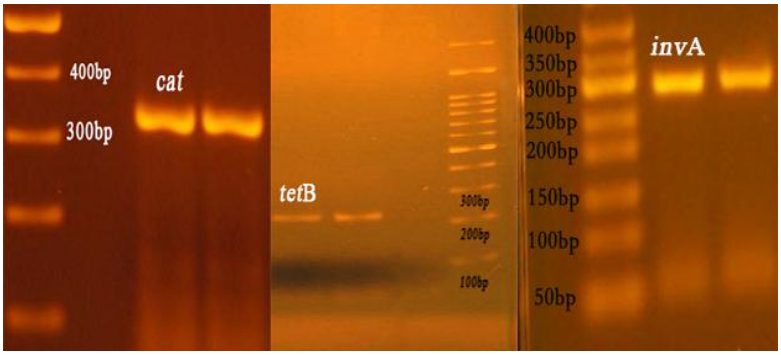

Fig. 1. Amplification of *inv*A, *tet*B and *cat* genes by PCR in NTS isolates: *inv*A gene amplicons (284 bp), *tet*B (227bp) and *cat* gene (349bp)

Table 2. Antimicrobial resistance of NTS isolates

Antibiotics	Resistant isolates N (%)
Total	22(100%)
Ampicillin	19 (86.4)
Tetracycline	17 (77.3)
Trimethoprim-sulphamethoxazole	17 (77.3)
Ceftazidime	11 (50)
Cefotaxime	7 (31.8)
Chloramphenicol	6 (27.3)
Streptomycin	7 (31.8)
Ofloxacin	6 (27.3)
Nalidixic acid	5 (22.5)
Amikacin	1 (4.5)
Ciprofloxacin	1 (4.5)
MDR	18 (82)
ESBL producers	4 (18)

MDR: Multiple drug resistance, ESBL: Extended spectrum β-lactamases

3.3 Molecular Detection of *tetB*, and *cat* Genes

Tetracyclin and chloramphenicol are still used as effective and cheap drugs in treatment of NTS infections. In the present study, *tet*B gene and *cat* gene were detected by PCR in 64.7% (11/17) of tetracycline resistant isolates and 50% (3/6) of isolates resistant to chloramphenicol, respectively (Fig. 1). The resistant genes were not detected both in sensitive isolates to tetracycline or chloramphenicol. These analyses agree with several studies which show that not all the resistant isolates have the genes *tetB* and *cat* genes, preventing the identification of NTS organisms based on these genes exclusively [42,43,44].

Table 3. Antibiotic resistant profile of the NTS isolates

Resistance pattern	Number of isolates
AM	1
CAZ, OFX	2
AM, SXT	1
AM, SXT, TE	4
AM, CAZ, TE, C	2
AM, TE, SXT, S	2
CAZ, CTX, SXT, C	1
AM, S, SXT, TE, C, NA	2
AM, CTX, CAZ, SXT, TE	1
AM, CTX, CAZ, OFX, SXT, TE	3
AM, CTX, CAZ, S, NA, SXT, TE	2
AM, AK, SXT, TE, C, S, OFX, NA, CIP	1

AM: Ampicillin, TE: Tetracycline, SXT: Sulphamethoxazole-Trimethoprim, CAZ: Ceftazidime, CTX: Cefotaxime, AK: Amikacin, S: Streptomycin, NA: Nalidixic acid OFX: Ofloxacin, CIP: Ciprofloxacin, C: Chloroamphenicol

4. CONCLUSION

This study revealed high prevalence of MDR non-typhoid *Salmonella* among the samples collected from patients with gastroenteritis in Minia Fever Hospital, Egypt, whichrepresents a serious health problem in the region under study. In addition, these patients might require more expensive drugs for effective treatment.Screening studies like this could help in determining effective treatment measures to control NTS infections in this region, and the data could also be used for future medical references. Quinolones and amikacin are the treatment of choice for the patients understudy. Not all the resistant isolates have the genes *tetB* and *cat* genes, which prevents the identification of NTS resistance based on these genes exclusivelytherefore more extensive research on the mechanisms of resistance by molecular studies is important for effective management of NTS infections not only in Egypt, but also worldwide.

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TRANSPARENCY DECLARATIONS

All authors have nothing to declare regarding this study. Also, they do not have any conflict of interest.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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