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RESEARCH ARTICLE

Molecular Detection of New Delhi Metallo Beta Lactamase 1 (NDM-1) - Producing *Salmonella* Typhi in Patients with Typhoid Fever

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Abstract

Background: Antibiotic resistance among different pathogenic bacteria becomes a major therapeutic change and requires prompt solutions. Aims: this study aimed to investigate the prevalence of New Delhi metallo-ß-lactamase-1 (*blaNDM-1*) gene in *Salmonella* Typhi isolated from patients with typhoid fever. Materials and Methods: This a cross-sectional study including 100 patients with typhoid fevers. A blood sample was collected from each patient, and cultivated on a selective media for *Salmonella* where the diagnosis was made phenotypically and by API20E system. Disk diffusion test was used to examine the microbial sensitivity to imipenem and meropenem, while the ability of the bacteria to produce the carbapenemase was examined using Modified Hodge's test. For *blaNDM-1*gene detection, the plasmid DNA was extracted and a specific set of primers was used to amplify this gene in conventional polymerase chain reaction (PCR). Results: Out of 100 patients, 37 (37%) had a positive blood culture, of which 13 isolates showed some antibiotic resistance: 7 isolates for imipenem, 4 isolates to meropenem, and 2 isolates both antibiotics. Of those 13 isolates, 10 (76.9 %) were positively showing their ability to produce carbapenemases, while 9 isolates (69.2%) showed a successful *blaNDM-1* gene amplification. Conclusions: There is a relatively high rate of carbapenemases-producing and as well as NDM-1 producing *S.* Typhi strains among Iraqi patients with typhoid fever

Introduction

Over the years, the emergence and widespread of resistant bacteria to different types of antibiotic around the world has become a major therapeutic challenge to doctors [1]. Antibiotic-resistant bacteria are achieving an emergency situation in some bacterial pathogens where few therapeutic alternatives remain and pan resistant strains are becoming more predominant [2].

Bacteria can become resistant to antibiotics through several mechanisms, such as preventing an antibiotic from entering the bacterial cell and actively exporting an antibiotic from the bacterial cell (that is, efflux of the antibiotic) [3]. *Salmonella* is a gram negative, rod shaped, facultative anaerobe, flagellated bacterium.

The epidemiology of *Salmonella* depends on the host preferences. The first group have confined as host-restricted serotypes that infect only humans such as *S.* Typhi. While the other group includes host-adapted serotypes which are related with one host species but can cause illness in other hosts such as *S. pullorum* in avian. The remaining serotypes are categorized into a separated third group [4].

Carbapenems are a group of ß-lactam antibiotics with a broad spectrum antibacterial activity. They include meropenem and imipenem, which are among the few therapeutic options used for treating Salmonellosis [5].NDM-1 is an enzyme that cleaves the amide bond of ß-

lactam ring and provides resistance against major classes ß-lactam antibiotics [6].New Delhi Metallo-ß-lactamase-1 gene (*blaNDM-1*) codes for NDM-1 [7].

When plasmid borne *blaNDM-1* disseminated through horizontal gene transfer (HGT), the possibility to treat infection in society becomes more difficult [8]. So, the goal of this investigation is to detect the presence of NDM-1 *Salmonella* producers in patients with typhoid fever.

Patients and Study Design

Over twelve months from November 2016 to November 2017, one hundred patients with typhoid fever were recruited in this cross-sectional study. They were 48 (48%) males and 52 (52%) females, the age rang was 18-40 year with mean (33.6±3.82). All participantents were outpatients or hospitalized in Abu-Graib Hospital/ Baghdad.

They were diagnosed by consultant physician from the clinical symptoms and laboratory findings. Blood sample and clinical data were collected from those patients. The samples were transported to the laboratory of Microbiology department in College of Medicine Al-Nahrain University. A consent letter was signed by each patient and the study was approved by the Research Ethical Committee College Medicine of Al-Nahrain University.

Blood Culture

Samples were immediately transferred to tryptic soya broth medium vial, which prepared specially for bacterial cultivation, and incubated in 37°C for 24 hr. Then samples were sub-cultured on XLD agar (OXOID, England). The initial reading was recorded after 24 hours and the results continued to be recorded for 72 hours. Negative broth cultures were incubated for five days and submitted for further sub-culture before reported negative. Positive cultures on XLD agar were diagnosed phenotypically and by API 20E system for Enterobacteriaceae to recognize the species level, according to the procedure suggested by the manufacturing company (bio-Merieux/France).

Antimicrobial Sensitivity Tests

Resistance patterns of *Salmonella* Typhi to imipenem and meropenem were studied by disk diffusion test (DDT) and minimum inhibitory concentration (MIC) after the incubation was completed. The zone of inhibition around the disks was measured and compared with the break points of clinical and laboratory standard institute (CLSI) [9]. The MIC was used to determine the lowest antibiotics concentration that inhibits growth of *S.* Typhi by a standard agar dilution method [10]. Two-fold dilutions from 512-0.5 µg/ml for two antibiotics were prepared from stock solutions at concentrations of 10 mg/ml, 1mg/ml, and 0.1 mg/ml, then Muller Hinton agar medium was prepared, sterilized by autoclaving.

After cooling, 25 ml of the medium were added to each antibiotic container; the content mixed well and poured into petridishes. The inoculum density was adjusted by using 0.5 McFarland standard tubes and then 20 microliters of each inoculum were spotted on the agar surface of Muller Hinton agar medium and incubated at 37^0 C for 24 hr. The stranded isolates from central public health laboratory *E.coli* ATCC25922 was used as a negative control.

Modified Hodge's Test (MHT)

For rapid detection of carbapenemases production by *S.* Typhi isolates, the MHT was used by preparing a 0.5 McFarland dilution of the *E. coli* ATCC 25922 as standard strain in 5 ml broth. Then these bacteria were streaked on plate of MH agar after which 10 µg meropenem susceptibility disks was placed in the center of the test area. *S.* Typhi was streaked in a straight line from the edge of the disk to the edge of the plate. The test plate was incubated for 24 hr. at 37 ^{0} C. When the Modified Hodge test was positive, result appear as clover leaf-like indentation [11].

Molecular Assays

Plasmid DNA extraction

The organisms were inoculated into 5 ml of Luria-Bertani broth and incubated for 20 hours at 37°C with shaking. Cells from 1.5 ml of an overnight culture were harvested by centrifugation at 17,310 × g a for 5 minutes. After the supernatant was decanted, the pellet was resuspended in 500 µl of distilled water. The cells were lysed by heating them at 95°C for 10 minutes, and cellular debris was removed by centrifugation. The supernatant was used as a source of template

for amplification and kept at deep freeze [12].

BlaNDM-1 Gene Amplification

A specific set of primers was used to amplify *blaNDM-1*gene in conventional PCR.

These primers were forward: 5'-GGGCAGTCGCTTCCAACGGT and reverse: 5'-GTAGTGCTCAGTGTCGGCAT that amplify 475bp internal fragment of the gene [13]. The PCR mix of 50 µl contained 100 ng total DNA as template, 1x PCR buffer with 1.5 mM MgCl 2, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25 U Ampli Taq DNA polymerase (Bioneer/Korea) and nuclease-free water to make up the final volume. The PCR conditions consist of an initial step of denaturation of DNA at 94 ^{0} C for 3 min, followed by 35 cycles of denaturation, annealing and extension at 94°C, 60°C and 72°C, respectively, for 45 s each. The final extension step was performed for 7 min at 72°C. The PCR product was subjected to 1% agarose gel electrophoresis in 1x tis borate EDTA buffer for 1.5 hr and was stained with ethidium bromide and visualized under a gel documentation system.

Results

Fifty-four (54%) of the included typhoid fever patients were males and 46 (46 %) were females. The female-to-male ratio was 1.10:1. The patient's ages ranged from 10 to 61 years old. The highest percentage group of patients were 41 to 50 years old (n= 24, 24.0 %) followed by 31 to 40 years old (n= 23, 23.0%) and the lowest percentage group of patients were 51-60 year old (n=13, 13%) (Table 1).Out of 100 patients, 37 (37.0%) had a positive blood culture. Of those, 7 isolates were resistant to imipenem and 4 isolates to meropenem, while 2 isolates resisted both drugs. The reminders (24) were sensitive.

Table 1: Distribution of Salmonella Typhi isolates according to age groups

Age groups	*Salmonella* Typhi Resistance	*Salmonella* Typhi Sensitive	Negative	Total
10-20	0	5	5	10
21-30	3	4	7	14
31-40	2	5	16	23
41-50	2	4	18	24
51-60	2	5	6	13
≥61	4	1	13	16
Total	13	24	63	100

Minimum Inhibitory Concentrations

The MIC of resistant *S.* Typhi was determined by using agar dilution method. An isolate was characterized as resistant if the MIC was greater than the breakpoint defined by clinical and laboratory standards institute (CLSI), while it will be as susceptible if it is below the breakpoint. MIC breakpoint for *S.* Typhi. Resistances have been establishing by the CLSI for each of imipenem and meropenem as ≥ 8µg /ml. The two isolates which were resistance to both imipenem and meropenem have an MIC 2—8 mg/mL (intermediate), while 11 isolates had MIC >8 mg/mL (resistant).

Modified Hodge Test

Result of this test showed that out of 13 carbapenem-resistant *S.* Typhi isolates, 10 (76.9 %) had the ability to produce carbapenemases. Regrettably, the test is unable to give any information on the type of carbapenemase enzyme produced by the isolates tested (Figure 1).

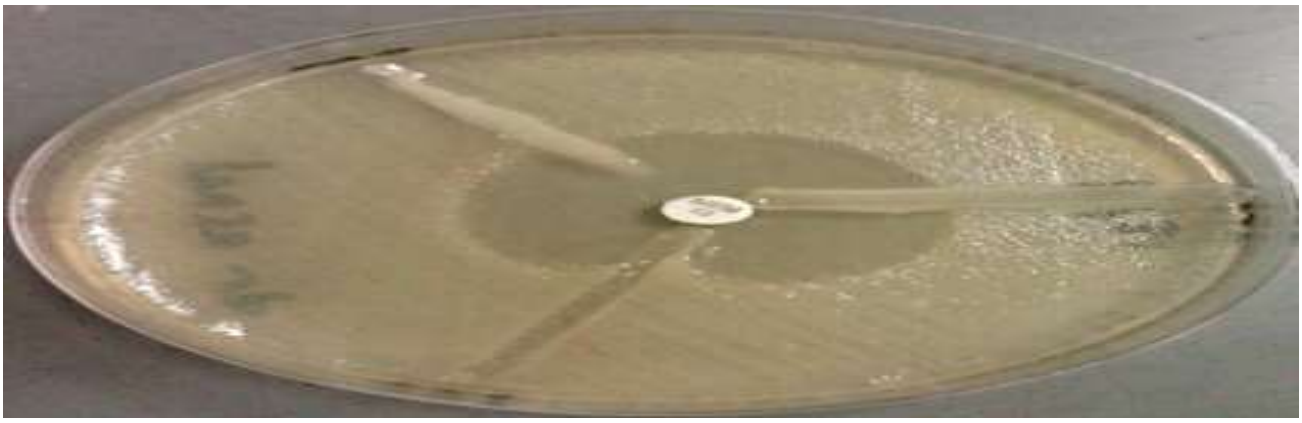

Figure 1: The Modified Hodge test (MHT) of carbapenemase production

PCR Screening for NDM-1 Encoding Gene

The result of *blaNDM-1* gene amplification showed a 475 bp fragment length which was detected in 9 (69.2%) of the 13 carbapenem-resistant isolates on plasmid DNA (Figure 2).

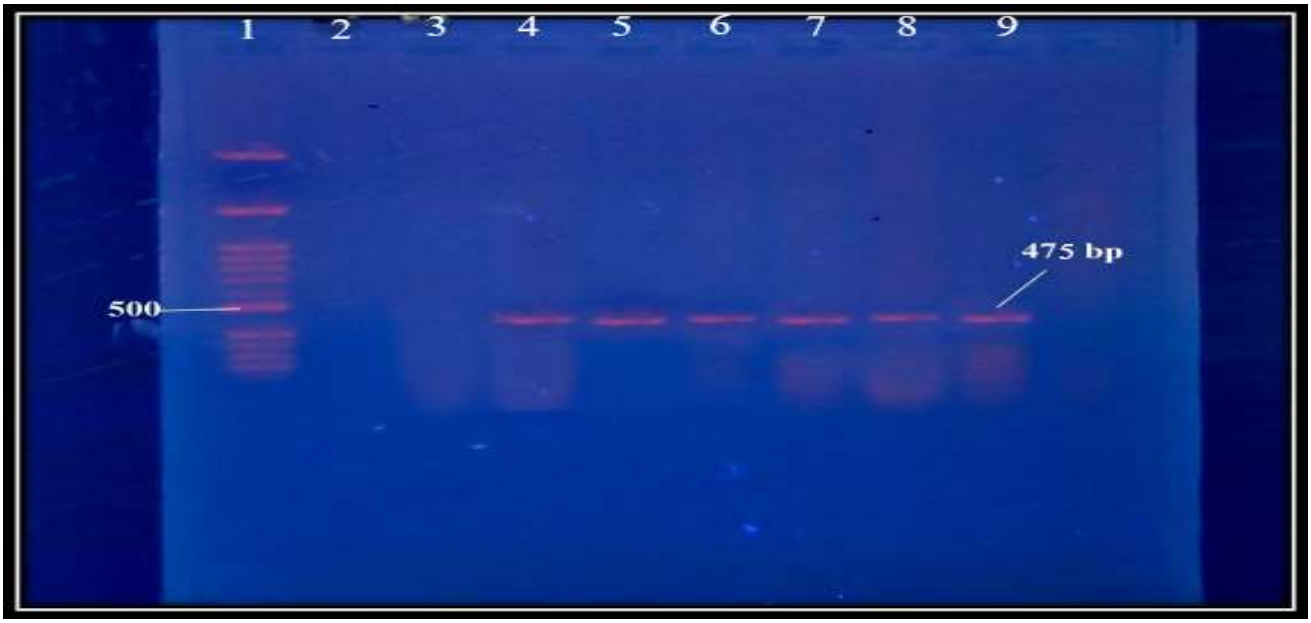

Figure 2: Gel electrophoresis (1% agarose, 7 v/cm², 1.5hrs) of bla-NDM1 gene (475 bp) amplified with specific primers. Lane 1: molecular marker, lane2: negative control, lane 3: negative isoltate, lanes 4-9: positive isolates

Discussion

In developing countries, typhoid fever is endemic and there was a wide spread of antibiotic resistance in *S.* Typhi mainly to chloramphenicol, amoxicillin, cotrimoxazole and ciprofloxacin which are the drugs of choice for treating typhoid fever. Even for carbapenem antibiotic, many researchers reported resistance to this antibiotic [14, 15, 16]. Thus, it is an urgent issue to determine the genes responsible for this resistance. The blood culture result in current study showed that 37 (37.0%) of patients were positive for *S.* Typhi. This rate is higher than that obtained by Beig *et al* [17]. Who found that blood culture yield 27.3% in India.

The possible explanation for such variation is the different sample size in each study. Furthermore, this difference between their results and ours can be attributed to patients' selection, study area as well as the method followed and the type of culture media used in the process of blood culture. Imipenem and meropenem have been used as an alternative therapy for the treatment of typhoid fever when there is no response to classical drug such as amoxicillin, ceftriaxone or even to fluoroquinolone. Regarding the MIC value for imipenem and meropenem, the current study showed that two isolates of *S.* Typhi had MIC 2-8 mg/mL (intermediate), while 11 isolates with MIC >8 mg/mL (resistant). These results disagree with that of a broad study done by Pokharel *et al* [18]. In Nepal who reported that all isolates of *S. enterica* serotype Typhi were susceptible to imipenem and meropenem. This might be giving evidence that the overuse and misuse of antibiotic including carbapenem groups in Iraq, as a country where antibiotics are sold over the counter. Jain *et al*. [19] reported that out of 344 *Salmonella* stains in India, only 2% have a resistance to 3rd generation cephalosporin. In this investigation, the presence of carbapenemases was evaluated in a 13 carbapenem-resistant *S.* Typhi isolates by using MHT.

Carbapenemases production may confer certain phenotypic characters to the organisms depending on the type of enzyme produced by species and the expression level of this enzyme, as well as presence of other resistance mechanisms [20]. However, there are some obstacles in the detection of carbapenemases. For some carbapenemases generating bacteria, the MIC of carbapenem is high but still within the susceptible ranges as well-defined by CLSI guidelines criteria [21].The use of simple and inexpensive screening procedure for detection of MBL producer bacteria is very important for infectious control measures to control the

spread of MDR isolates particularly in hospitalized patients.

In the current study, out of 13 *S.* Typhi isolates, there were 10 (76.9%) isolates which were positive for MHT which optimistically indicates that they may possess the carbapenemase genes that mediate the carbapenem resistance. Some researchers stated that sensitivity and specificity of the MHT was 100% [22]. However, the test has some disadvantages such as the difficulty in interpretation and the impossibility to identify the class of the present carbapenemases [23].

Other reports have also found false negative or positive results when MHT was used to screen carbapenemases in Enterobacteriaceae. The false positive MHT probably results from low-level carbapenem hydrolysis by extended-spectrum ß-lactamase, ESBLs [24, 25].

According to the present results, *blaNDM-1* gene was detected in 9 (69.2%) out of 13 carbapenem-resistant *S.* Typhi. This high rate of resistance to imipenem and meropenem provides evidence that the overuse and misuse of carbapenem antibiotics lies extensive burden on the treatment of typhoid fever problem in this country, because it limits therapeutic options for treating this infection. Moreover, these results may also alert the overuse of antibiotics in animal food which in turn transfers to humans by meat consumption. In this regard, ESBL-producing *Salmonella* was detected in several countries such as France, Italy, India, Pakistan, Nepal and Egypt [26]. However, to the best of our knowledge this is the first study describe an infection caused by *S. enterica* serotype Typhi ESBL-producing strains. This type of bacteria is considered an emerging threat because it gained ability to hydrolyze carbapenem antibiotics which are the drugs of choice for the treatment of patient with typhoid fever.

In conclusion, these data indicate the high rate of carbapenemases- producing and as well as NDM-1 producing *S.* Typhi strains among Iraqi patients with typhoid fever. Every measure should be adopted to prevent the random massive use of different antibiotics.

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Ethical Clearance

The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

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