Frequency of blaPER-1 Extended-spectrum β-Lactamase Gene among Different Salmonella enterica serovar Typhi Isolated from Human and Chicken Meat Samples

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Abstract: Species of Salmonella are able to form biofilms on biotic and abiotic surfaces. This study was aimed to survey the presence of blaPER-1 gene in clinical and food origin Salmonella enterica serovar typhi (S. typhi) isolates and it is relation to biofilm formation. Through a period prolonged from June to September, 2016, (10) clinical S. typhi isolates were isolated from blood specimens collected from hospitalized typhoid fever patients at a hospital in Baghdad while (10) S. typhi isolates were isolated from chicken samples. The isolates ability to form biofilm was assayed using the tissue culture plate and Congo-red agar methods, and the results revealed that 18 strains were biofilm producers. Susceptibility of all clinical and food origin S. typhi isolates (20) toward different antibiotics were tested and results showed a high level of resistance by both clinical and food origin isolates. All the strains were tested for phenotypic ESBL production and it was found that 16 isolates (80%) were ESBL producers. PCR technique was performed to detect the presence of blaPER-1 gene among both clinical and food origin strains and our findings was that eleven (52%) were contained blaPER-1 gene.

Keywords: blaPER-1, S. typhi, chicken meat, ESBLs, Biofilm.

1. Introduction

Salmonella enteric serovar Typhi (S. typhi) is the main Salmonella serovar that causes typhoid fever which is a major public health problem in developing countries (Qamar et al., 2014), that is responsible for 21 million new infections yearly which give rise to more than 200,000 deaths worldwide (Crump et al., 2004).

Poultry birds have recurrently been implicated in salmonella contamination and hence being the chief origin of this bacteria in humans (Baeunler et al., 2000). The holding pens represented the contamination niche before slaughter, also the technical applies raised the salmonellosis incidence (Kent et al., 1981; D’Aoust, 1994). An increased surface contamination resulted from meat dressing after slaughter, thus contamination level enlarging as twenty percent (Forsythe and Hayes, 1998). In many countries, Salmonellosis considered as an important food poisoning causes health and economic loses because of these improper practices prior, during and after slaughtering (Rumeu et al., 1997). The incriminated foods that implicated in salmonellosis are beef, poultry, eggs, pork, and milk (Gomez et al., 1997). When Salmonella infection extends beyond the intestine, suitable antimicrobial treatment can be useful in saving life (Hohmann, 2001; Shah and Korejo, 2012).

Extended-spectrum cephalosporins are the typically used antimicrobial agents to heal infections (ceftizidime, ceftriaxone, cefotaxime, and ceftipime), because of their strong bactericidal activity, low toxicity and broad spectrum (Reinert et al., 2007). Multiresistant Salmonella strains of different serovars are increasingly common and have become a problem of worldwide attention (Olsen et al., 2001).

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated enzymes that can hydrolyze the broad spectrum beta-lactams which they are transmitted by plasmids among different strains of bacteria (Chen et al., 2015). Salmonella spp. have been reported to express different types of extended-spectrum β -lactamases (ESBL) types such as SHV, PER, TEM, CTX-M and OXA enzymes (Ogunleye and Carlson, 2011). One major factor sharing in antibiotics resistance is the biofilm formation ability of the bacteria on animate and inanimate (Chen et al., 2014).

The PER-1 type Extended-spectrum β–lactamase is a class A enzyme which granting high-level of resistance to antibiotics of beta-lactams (Endimieni et al., 2006). This study aimed to was to detect the presence of blaPER-1 gene in S. typhi isolated from clinical and chicken samples and also study the relationship of the presence of blaPER-1 gene and biofilm formation.

2. Materials and Methods

Bacterial isolation and Identification

Twenty clinical and food origin S. Typhi isolates were involved in the current study; ten of which were clinical isolated from blood specimens collected from hospitalized typhoid fever patients at a local hospital while the other (10) S. typhi were isolated from imported frozen chicken meat
samples that were randomly collected from supermarkets in Baghdad, through a period extended from June to September, 2015. Depending the instructions of the Iraq Standard Criterion No.3/2270 in Isolation, Enumeration and Identification of Microbiological Groups in Foods, (C.O.S.Q.C. 2006). Salmonella was isolated from these food samples using Xylose LysinDeoxycholate (XLD) agar and Salmonella-Shigella (S.S) agar. The isolates identified at species level by Vitek-2 system (Bio-Merieux, France), by using ID-GNB cards according to the manufacturer's instructions.

**Antibiotic susceptibility test**
Kirby-Bauer method was carried out to detect the antimicrobial sensitivity of all S. typhi strains toward different antimicrobial agents as described by WHO, (WHO, 2003) and the antibiotics tested in the current study are listed in table (2).

<table>
<thead>
<tr>
<th>Antimicrobial discs</th>
<th>Code</th>
<th>Disc potency (µg/disc)</th>
<th>Manufacturing Company/ Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime - Clavulanic acid</td>
<td>CUG</td>
<td>30/10</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Amikacin</td>
<td>AK</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Ampicillin-Sulbactam</td>
<td>SAM</td>
<td>10/10</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Cefepime</td>
<td>CPM</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>CAZ</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CTX</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>CIP</td>
<td>5</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>CZ</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>FOX</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>GM</td>
<td>10</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Temocillin</td>
<td>TEM</td>
<td>30</td>
<td>Bioanalyse/ Turkey</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>TN</td>
<td>10</td>
<td>Bioanalyse/ Turkey</td>
</tr>
</tbody>
</table>

**Detection of the phenotypic ESBL production**
Both food and clinical S. typhi isolates were detected for phenotypic ESBL production using NCCLS confirmatory test (NCCLS, 2000), in this test the bacterial strain was swabbed on to a Mueller-Hinton agar plate. Ceftazidime (30 µg) and ceftazidime + clavulanic acid (30/10 µg) were put on the agar surface; after incubation, an increase as 5mm in diameterzone of ceftazidime + clavulanate disc and that of ceftazidime disc alone, reflecting ESBL production.

**Genotypic Detection of blaPER-1 geneby PCR assay**
Polymerase chain reaction (PCR) assay was used for genotypic detection of blaPER-1 gene in all clinical and food origin isolates that showing ESBL positive by phenotypic detection.

**Extraction of the bacterial DNA**
Bacterial DNA was extracted from all clinical and food-origin isolates by suspending 2-3 colonies of each strain under study which grown on MacConkey agar plates in 500 µl of nuclease-free water (Promega, USA) and heating by using a water bath at 90°C for 10 min. Samples were spun at 10000 rpm for 10 min. These samples were used as DNA template for PCR assay (Endimiani et al., 2009).

**Amplification reaction**
The blaPER-1 Forward primer: 5’- ATGAAT GTCATTATAAAAGCT -3’ and Reverse primer: 5’-TTA ATT TGG GCT TAG GG-3’ (Alpha DNA, Canada) were used and PCR mixture was set up in a total volume of 25 µl (for each isolate) included: 12.5µl of Master mix 2X (Promega, USA), nuclease-free water (4.5µl), DNA sample (5µl), blaPER-1 Forward primer (1.5µl) and Reverse primer (1.5µl). Temperature cycling conditions included an initial denaturation at 94°C for 5 min thereafter 45 seconds at 94°C for 30 cycles, 30 seconds at 52°C as annealing and 30 seconds at 72°C as extension. Then 7 min at 72°C as a final extension (Kalantar et al., 2012).

**Agarose gel electrophoresis**
Gel electrophoresis was used to visualize the PCR products with the aid of SYBR-Green and UV trans-illuminator documentation system.

**Biofilm formation assay**
All clinical and food origin S. typhi were subjected to biofilm production.

**Congo-red agar assay**
Congo-red agar (CRA) was used to detect both clinical and food-origin Salmonella isolates ability to produce slime layer and form biofilm (Freeman et al., 1989). The bacterial isolates were streaked on CRA and were incubated at 37°C for 24-48 h aerobically. Slime layer producers grown as dry black crystalline colonies, while pink colonies indicated the negative result, an indeterminate result was dark colonies without dry crystalline colonial morphology.

**Tissue culture microtiter plate assay**
A previous study described Tissue culture microtiter plate method as a quantitative test (Christensen et al., 1995). It is considered as the gold standard technique for biofilm detection. Trypticase soy broth (TSB) supplemented with 1% glucose w/v was inoculated with fresh Salmonella isolates and incubated at 37°C for 24 h. A fresh medium was added to the culture in a proportion of 1:100 in the wells of 96 well flat bottom polystyrene tissue culture plate, few wells filled with sterile broth only, these considered as negative control, The next step was the incubation of the plates at 37°C for 24 h, then after incubation, the plates were washed by tapping gently to diminish planktonic bacterial cells. Crystal violet (0.1%) w/v was used to stain the biofilm formed by bacteria that sticking to the wells, thereafter a washing step by distilled water was applied to remove excess crystal violet stain, then the plate were air dried. Micro ELISA auto reader (model 680, Biorad, UK) at wavelength 630 nm was used to measure the opticaldensity (OD) of stained wells. (Table2).
Statistical Analysis
In order to compare between biofilm formation, antibiotic resistance and PER-1 gene harbouring versus positive and negative number, using Chi-Square test (Exact Fisher test). Get result by online program for exact Fisher test (2*2), P-value=0.0014, that is significant differences at p≥0.05.

3. Results

Bacterial isolates
Ten clinical S. typhi were isolated from blood specimens of hospitalized typhoid fever patients and ten S. typhi were isolated from imported frozen chicken meat samples that were collected from markets in Baghdad randomly using Xylose Lysin Deoxycholate (XLD) agar and Salmonella-Shigella (S.S) agar; identification of the twenty strains at species level was done by Vitek-2 system (Bio-Merieux, France), by using ID-GNB cards according to the manufacturer's instructions (Figure 1).

Figure 1: Salmonella colonies with black center on S.S agar after 24 h incubation at 37˚C

Biofilm Formation
Using both tissue culture plate (TCP) and Congo-red agar (CAR) methods, the ability of isolates to form biofilm was assayed. The highest strong biofilm formation ratio was by food origin isolates (83.33%) in CRA method while clinical isolates were all of them biofilm producers in TCP method; (Table 3, Fig 1).

Figure 2: Left: Crystal violet stained wells representing biofilm formation by S. typhi isolate using TCP method. Right: Congo red agarelicits black glistening colonies of S. typhi isolated from chicken meat upon 24 h of aerobic incubation at 37˚C

Testing the Antibiotic susceptibility results
All clinical and food origin S. typhi (20) were tested for susceptibility toward different antibiotics; ciprofloxacin, cefazolin, ampicillin/sulbactam, ceftazidime/clavulanicacid, cefepime, ceftazidime, amikacin, cefotaxime, cefoxitin, gentamicin, temocillin and tobramycin were tested by Kirby-Bauer method, and the obtained results shows a high level of resistance by both clinical and food origin isolates to the tested antibiotics.

Genotypic Detection of blaPER-1 gene
In this study, all clinical and food origin S. typhi isolates were subjected to monoplex PCR technique for the purpose of blaPER-1 gendetecion. Among all clinical and food origin S. typhi eleven (52%) were contained blaPER-1 gene; 6 clinical and 5 food origin S.typhi isolates that amplified the blaPER-1 gene and elicited 927 bp bands after being electrophoresed using 1.5 % agarose gel electrophoresis (Figure 3).

Phenotypic detection of ESBL
Twenty clinical and food origin S. typhi isolates were tested for ESBL production and the results showed that 16 isolates (80%) were ESBL producers (Table3, 4).
Various genetic elements from food origin strains may accept the resistance genes via horizontal gene transfer, reflected high risk for human salmonellosis treatment. These results were similar to our results, whereas other studies have obtained results concerning genotypic ESBL detection of S. typhi strains were resistant to more than 3 of the tested antibiotics. Out of 20 clinical and food origin S. typhi isolates, 18 strain were biofilm producers using both TCP and Eliza reader.

Relation between antibiotic resistance and biofilm formation ability was recorded previously; and it is a vital subject to be focused that Salmonella ability to adhere to food surfaces and form biofilms which may offers a significant procedures to eradicate this pathogen from food and thus reducing salmonellosis in human.

Table 3: Clarify the results of all tested isolates for biofilm formation, antibiotic susceptibility, phenotypic and genotypic ESBL detection

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Antibiotic Resistance</th>
<th>Biofilm formation (Eliza reader)</th>
<th>Phenotypic ESBL production</th>
<th>PER-1 gene presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>+</td>
<td>0.098</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S2</td>
<td>+</td>
<td>0.105</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S3</td>
<td>+</td>
<td>0.063</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S4</td>
<td>+</td>
<td>0.127</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S6</td>
<td>+</td>
<td>0.092</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S7</td>
<td>+</td>
<td>0.100</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S8</td>
<td>+</td>
<td>0.098</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S9</td>
<td>+</td>
<td>0.085</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S10</td>
<td>+</td>
<td>0.115</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S11</td>
<td>+</td>
<td>0.097</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S12</td>
<td>+</td>
<td>0.113</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S13</td>
<td>+</td>
<td>0.094</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S14</td>
<td>+</td>
<td>0.131</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S15</td>
<td>+</td>
<td>0.155</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S16</td>
<td>+</td>
<td>0.096</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S17</td>
<td>+</td>
<td>0.092</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S18</td>
<td>+</td>
<td>0.088</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S19</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S20</td>
<td>+</td>
<td>0.101</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: S1-S10: Clinical isolates; S11-S20: Food origin isolates

*Significant differences at \(P\geq0.05\).

4. Discussion

In this study, we obtained9 (56.25%) positive ESBL phenotypes strains of clinical S. typhi while 7 (43.75%) positive ESBL phenotypes strains of food origin S. typhi. Whereas only one clinical and 3 food origin S. typhi were negative (non-ESBL) phenotype.

Genotypic detection of blaPER-1 gene in this study revealed eleven (52%) of both clinical and food origin S. typhi strains were contained this gene as 6 (54.54%) and 5 (45.45%) strains respectively. Another study showed such results (Akhi et al., 2012). This high ESBL production among clinical strains may be due to improved phenotypic procedure used in treatment. Mirsalehian et al., 2010 obtained results concerning blaPER-1 gene harboring which were similar to our results.

Regarding food origin strains, this abundant resistance reflected high risk for human salmonellosis treatment. These food origin strains may accepted the resistance genes via various genetic elements from clinical strains (Goncagul et al., 2004). All the tested S. typhi strains were resistant to more than 3 of the tested antibiotics. Out of 20 clinical and food origin S. typhi; 18 strain were biofilm producers using both TCP and CRA assays. Several studies revealed that Salmonella can simply attach to various food-contact surfaces and form biofilms (Chia et al., 2009; Marin et al., 2009; Rodrigues et al., 2011).

References


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553