

Frequency of *bla*_{PER-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 *bla*_{PER-1} gene in clinical and food origin *Salmonella enterica* serovar *typhi* (*S. typhi*) isolates and its 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 *bla*_{PER-1} gene among both clinical and food origin strains and our findings was that eleven (52%) were contained *bla*_{PER-1} gene.

Keywords: *bla*_{PER-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 (Baeumler *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 losses 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 (ceftazidime, ceftriaxone, cefotaxime, and cefepime), 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 detect the presence of *bla*_{PER-1} gene in *S. typhi* isolated from clinical and chicken samples and also study the relationship of the presence of *bla*_{PER-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 Iraqi 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 2: The tested antimicrobial discs

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

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 diameter zone of ceftazidime + clavulanate disc and that of ceftazidime disc alone, reflecting ESBL production.

Genotypic Detection of *bla*_{PER-1} gene by PCR assay

Polymerase chain reaction (PCR) assay was used for genotypic detection of *bla*_{PER-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 *bla*_{PER-1} Forward primer: 5'- ATGAAT GTCATTATAAAAAGCT -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), *bla*_{PER-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 sterile 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 optical density (OD) of stained wells. (Table 2).

Table 2: Interpretation of Biofilm Formation

OD value average	Production of biofilm
≤ OD /ODc < ~ ≤ 2x ODc	Non / weak
2x ODc < ~ ≤ 4 x ODc	Moderate
> 4x ODc	Strong

Optical density cut- off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control (Hassan *et al.*, 2011).

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 $p \geq 0.05$.

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)

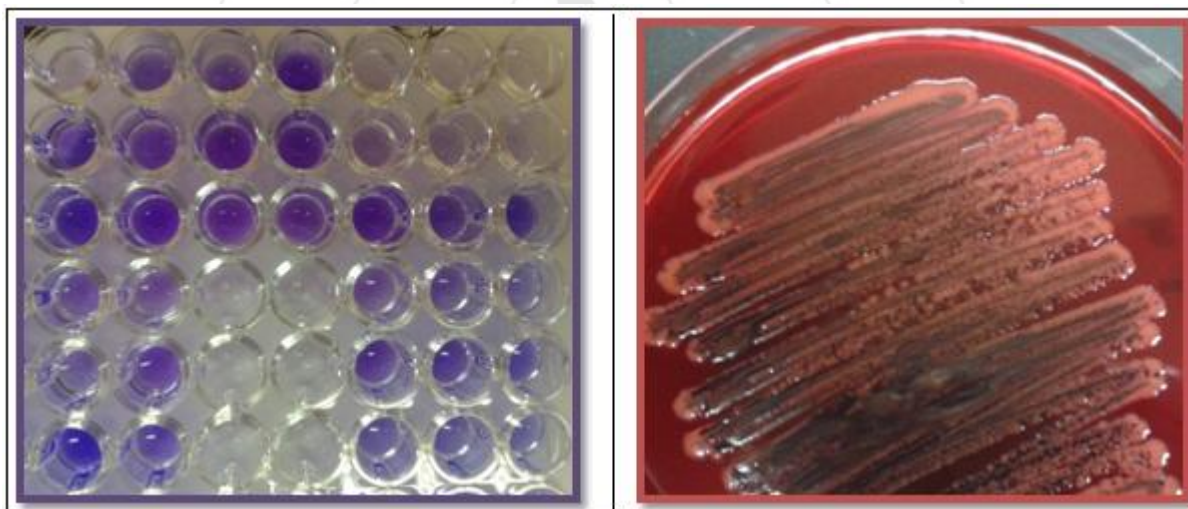


Figure 2: Left: Crystal violet stained wells representing biofilm formation by *S. typhi* isolate using TCP method, Right: Congo red agar showing black glistening colonies of *S. typhi* isolated from chicken meat upon 24 h of aerobic incubation at 37°C

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/clavulanic acid, cefepime, ceftazidime, amikacin, cefotaxime, ceftazidime, 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.

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 (Table 3, 4).

Genotypic Detection of bla_{PER-1} gene

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

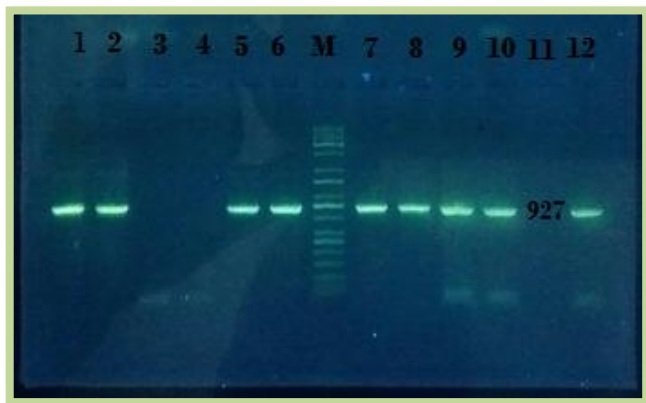


Figure 3: Amplified PCR products of PER-1 gene (927 bp) of *S. typhi* strains in monoplex polymerase chain reaction (PCR)

Sybr-green stained 1.5% agarose gel electrophoresis: M: The DNA molecular weight marker (1000bp ladder "Kapa"); Lane 1: Lane 2, Lane 5, Lane 6, Lane 7, Lane 8, Lane 9, Lane 10 and Lane 12: Positive amplification of 927 bp for *bla*PER-1 gene of the strains S1, S2, S4, S7, S10, S11, S14, S15 and S20 respectively; Lane 3, Lane 4 and Lane 11: *bla*PER-1 Negative strains S5, S9 and S 19 respectively.

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

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

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

Table 4: Comparison between Biofilm formation, Per-1 gene harbouring with phenotypic expression and Antibiotic Resistance by *S. typhi* isolates

Total <i>S. typhi</i> isolates No.	Biofilm formation	Phenotypic expression (Antibiotic Resistance)	ESBL Production	PER-1 Gene harbouring	P value of X ² -Test value
Positive No. 20 (100%)	18	20	16	11	0.0014
Negative No.	2	0	4	9	

*Significant differences at $P \geq 0.05$.

4. Discussion

In this study, we obtained 9 (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 *bla*PER-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 *bla*PER-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).

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 biofilm formation, which may offers a significant procedures to eradicate this pathogen from food and thus reducing salmonellosis in human (Giaouris *et al.*, 2012).

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