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Original article

Molecular detection of *ompX* gene related to immune resistance in *Salmonella typhi*

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ABSTRACT

Background: *Salmonella* is known for its ability to invade and survive within host cells, which is attributed to various virulence factors it possesses. These virulence determinants enable the bacteria to adhere to and invade host cells, bypass host immune defenses, and replicate within host cells, leading to the establishment of infection. The objective of this study was to isolate and identify *S.enterica* through PCR and detect the *ompX* gene in clinical samples from human subjects. **Materials and Methods:** A total of 75 blood samples were collected from patients with food poisoning and diarrhea between June 2021 and March 2022 at Al-Diwaniyah General Hospital and from some patients under internal medicine care. Identification procedures included culturing, utilizing the Vitek-2 system, and confirmation through PCR and DNA sequencing analysis. **Results:** The isolates were distributed as follows: 10 (33.3%) for *S. typhimurium*, 8 (26.6%) for *S. typhi*, and 12 (40%) for *S. paratyphi B.* The molecular detection of the outer membrane gene (*ompX*) for *Salmonella enterica serovar* was conducted using conventional PCR, revealing a high prevalence of the *ompX* gene in 30 (100%) isolates. The in-silico analysis identified a missense mutation in the isolates within the *ompX* gene, leading to the replacement of certain amino acids. This substitution encompassed both conserved and non-conserved types of amino acids. These genetic variations were further supported by the construction of a phylogenetic tree, which illustrated the evolutionary relationships among these proteins and highlighted the genetic diversity present in the *ompX* gene. In **conclusion:** It is indeed concerning that virulence genes in *Salmonella* strains could be enhancing their pathogenicity and potentially leading to more widespread disease outbreaks. Monitoring and understanding the presence and expression of these virulence genes are crucial in order to effectively manage and mitigate the risk of such outbreaks.

Introduction

Gram-negative bacilli known as *Salmonella* infect and colonize people, resulting in a variety of clinical signs, such as bacteremia, enteric fever, and gastroenteritis. *Salmonella typhi* is the strain that causes enteric fever; other *Salmonella* strains are referred to as non-typhoidal *Salmonella*. The *Enterobacteriaceae* family of bacteria includes the *Salmonella* bacterium. *Salmonella enterica* and *Salmonella bongori* are the two primary forms of these bacteria according to the present categorization; permission for the third type, *Salmonella subterranean*, is still pending [1]. *Salmonella enterica* is frequently responsible for food and waterborne infections, leading to various clinical illnesses in both humans and animals. The

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immune response to *Salmonella* requires a coordinated effort of different immune mechanisms that act swiftly to reduce bacterial levels. Despite this, *Salmonella* has evolved multiple tactics to evade and manipulate the host's immune responses, enhancing its ability to cause infection and thrive within the host [2, 3]. *Salmonella* serotypes and strains can exhibit a wide range of disease severity upon infection, with certain serovars being more commonly linked to severe invasive illness in humans, while others tend to cause milder cases of gastroenteritis [4]. Around 2700 distinct serovars of *Salmonella* spp. have been identified. These serovars are responsible for a wide range of diseases, including typhoid fever (enteric or systemic), which is caused by *S. typhi* and *S. Paratyphi* A, and gastroenteritis (intestinal or diarrheal) caused by nontyphoidal *Salmonella* (NTS) (*Salmonella. typhimurium and Salmonella. enteritidis*) [5, 6]. Thus, among populations living in big cities and rural areas with inadequate sanitation and little access to clean water, enteric fever continues to be a serious public health concern [7]. The outer membrane proteins (Omps) are conserved proteins found in gram-negative bacteria that show immune reactivity. Some Omps have been isolated and utilized as antigens, with a few of them demonstrating immune potential [8,9]. Food contamination is a common way for this bacterium to infect humans. The pathogenicity of *Salmonella* is determined by the existence of several virulence factors, which are encoded on *Salmonella* pathogenicity islands (SPIs). Due to the sensitivity, specificity, and speed of nucleic acid amplification, sophisticated molecular approaches have recently been developed for the identification of foodborne microorganisms [10]. Thus, the purpose of this investigation was to isolate and identify *Salmonella. enterica* from human clinical samples. Furthermore, the characteristics and its molecular virulence traits are detected by PCR and sequencing.

Materials and methods

Samples Collection and Processing

Between June 2021 and March 2022, a total of 75 blood samples were drawn from patients who visited Al-Diwaniyah General Hospital with food poisoning and diarrhea, as well as some patients who were seen by internal medicine physicians. The isolates were identified using biochemical reactions after being inoculated on both selective and differential medium, in compliance with the diagnostic protocols suggested by Forbes *et al*. [11]. Every specimen was streaked on blood agar, bismuth sulfite agar, Ramba QUICK *Salmonella* broth, Macconkey agar, *Salmonella-Shigella* agar, and CHROM agar. The samples were then incubated for a whole day at 37°C in an aerobic environment. The Vitek-2 system (BioMe´rieux, France) performed confirmatory identification in accordance with the manufacturer's instructions. DNA sequencing analysis and Polymerase Chain Reaction (PCR) were used for additional confirmation.

Extraction of Genomic DNA

A Genomic Extraction Kit made by the American company Geneaid was used to extract the DNA from the isolates of *Salmonella* under study. Following the company's directions, the extraction procedure was completed. With a Nanodrop spectrophotometer, which reads absorbance at a wavelength between 260 and 280 nm to determine nucleic acid content (ng/µL), the purity of the extracted nucleic acid was examined.

Detection by Polymerase Chain Reaction (PCR)

PCR amplification was done using a conventional thermal cycler and was performed according to the conditions in **Table 1**.

Using specific primers according to the mentioned conditions, as illustrated in **Table 2**.

DNA sequencing

Following PCR confirmation of the identified gene, DNA sequencing analysis was utilized to ascertain the genetic sequence of the genetically encoded gene (*ompX*). The results of the PCR procedure were forwarded to Macrogen, a Korean company, for genetic sequencing using the DNA sequencing system. The National Center for Bioinformatics and Technologies' BLAST tool was used to analyze the target gene (*ompX*) results. The NCB-Gen Bank Submission was then used for scientific registration. The phylogenetic tree of outer membrane proteins was created using the MEGAX program, which was also used to calculate the genetic distance between the proteins in local isolates and standard strains in order to determine the genetic dimension of the target gene.

Statistical analysis

A statistical software program (IBM, SPSS V.23) was used to experiment. Statistics were used to characterize the data in terms of relative frequencies (percentages) and frequencies (number of cases). At $p \leq 0.01$ and $p \leq 0.05$, the least significant Pearson correlations were found.

Ethical approval

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. This was performed with the patient's verbal and analytical approval before taking the sample. To get this approval, the study protocol, subject information, and consent form were reviewed and approved by a local ethics committee according to document number 118 0\2022.

Results

Identification of bacterial isolates

Twenty-one isolates were obtained from blood and nine isolates from stool. A total of 30 pre-identified *Salmonella enterica serovars* isolates were identified at the subspecies level into (12) isolates as *S. paratyphi B,* (9) isolates as *S. typhimurium*, and(8) isolates as *S. typhi*. The identification was done according to the following results.

Macroscopic features

The morphological and biochemical identification of *S. enterica* was established; On MaCconkey Agar (MAC) the results showed that *Salmonella* species were none lactose ferment, smooth, colorless colonies; while on nutrient agar the isolates were small, smooth, rounded and pale as well as all *Salmonella* isolates were appeared white and non-hemolytic, smooth white or a Pale colonies on blood agar.

On *Salmonella-Shegiella* agar plates, the isolates appeared as pink colonies, black edges, H2S forming; also appeared as green colonies with black center on bismuth Sulfite agar, and as pink colonies on CHROM agar, while causing turbidity of culture in the RambaQUICK *Salmonella* broth. All these media were used to confirm *S. enterica* isolates, as shown in **Figure 1**.

Identification of *S.enterica* isolates using the Vitek®2system.

The manual identification of *Salmonella enterica* isolates was confirmed by the Vitek®2-automated system. The results showed that *Salmonella enterica* isolates were distributed based on different serotypes as follows: 12 isolates for *S. enterica* serotype *paratyphi B*, 10 isolates for *S. enterica* serotype *typhimurium* 8 isolates for *S. enterica* serotype *typhi*. The probability of identification ranged 95% -99% as shown in **Table3**.

Molecular characterization of *S. enterica* isolates

The *ompX* gene was investigated in this study, which played a significant role in the pathogenesis of *Salmonella enterica*. This gene was established by the conventional PCR technique.

Detection of *ompX* by PCR.

The outer membrane protein X gene (virulence plasmid gene) was pre sentinel isolates of *Salmonella enterica* 30(100%).

Genetic analysis of outer membrane proteins of *S. enterica serovars*

Outer membrane protein (ompx)

The ompX protein had a role in the resistance against attack by the human complement system. The results of the genetic analysis of the ompX protein sequence showed that the percentages of identity of local *S. enterica serovars* isolates with standard *S. enterica* strains from NCBI ranged from (98.83-100%), while the percentages of genetic variation ranged from (0.0-1.17%). Isolates No. 9, 17, 27, and 29 did not contain any genetic variations, as the percentage of identity was 100% between them and the standard strain, as shown in **Table 4**.

Genetic analysis of the ompX protein sequence showed that genetic variation to the ompX protein occurred only in *S. paratyphi B* isolates No.5 and 7, where glycine was converted to alanine and isoleucine to leucine, and this was a conserved substitution because the amino acids carry the substituent bearing the same physicochemical properties. When comparing it with the phenotype for serum resistance found that these two isolates were highly resistant to most blood groups.

Figure 3 shows the convergence between local and standard strains from the NCBI by drawing the phylogenetic tree. Using the UPGMA approach, the evolutionary history was deduced. It displays the ideal tree. Beside each branch is the proportion of replicate trees where the corresponding taxa clustered together in the bootstrap test (1000 repetitions). The phylogenetic tree is depicted accurately, with branch lengths measured in evolutionary distances that correspond to the same units.

The units of the evolutionary distances, which are the number of amino acid substitutions per site, were determined by applying the Poisson correction method. There were nine amino acid sequences in this investigation. For every sequence pair (pairwise deletion option), all uncertain

locations were eliminated. The completed dataset included 171 positions in the final dataset.

Table 1. PCR thermos cycling conditions.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
ompX	95C^0 /2 min	95C^0 / 30 sec	57.3 C^0 /30sec	72 C^0 /1 min	72 C^0 /5 min	35

Table 2. Primer sets used for the detection of the *ompX* gene polymorphism.

Gene	Primers	Product size (bp)	Source/origin	References
ompX	F- CCCCGAAAGGCGGATTTTTC R-CGCCCTTAGACACCGTGAAT	**644 bp**	**IDT\ USA**	**Designed**

Table 3. Distribution of *Salmonella enterica* isolates.

Serotyping	No. of isolates%
S. paratyphi B	12(40%)
S. typhimurium	10(33.3%
S. typhi	8(26.6%)
Total	30(100%)

Table 4. The NCBI homology sequence identity of the ompX protein between local *Salmonella enterica serovar* and NCBI-BLAST submitted related *S. enterica* strains.

S. enterica serovar	**Nucleotide polymorphism**	**Amino acid polymorphism**	**Type of polymorphism**	**Percentage of variations**	**Percentage of identity**
S. paratyphi BisolateNo.5	GGC>GCG ATT>CTG	G>AI>L	Missense Missense	1.17	98.83
S. paratyphi Bisolate7	GGC>GC GATT>CTG	G>AI>L	Missense Missense	1.17	98.83
S. typhimurium isolate 9	None	None	None	0	100
S. typhimurium isolate 17	None	None	None	0	100
S. typhi isolate 27	None	None	None	0	100
S. typhi isolate 29	None	None	None	0	100

*I: Isoleucine, L: Leucine, G: Glycine, A: Alanine

Figure 1. A: *S .enterica* non-lactose fermentation on MacConkey agar. **B:** *S. enterica* appears as pink colonies with CHROM agar. **C:** *S. enterica* appears as a green colony with a black center on Bismuth Sulfite agar. **D:** *S. enterica appears* white and non-hemolytic, smooth, white or Pale colony on blood agar. **E:** *S. enterica* appears black colony on *S.S* agar. **F:** *S. enterica because of* the turbidity of culture in the Ramba QUICK *Salmonella* broth (CHROM broth**).**

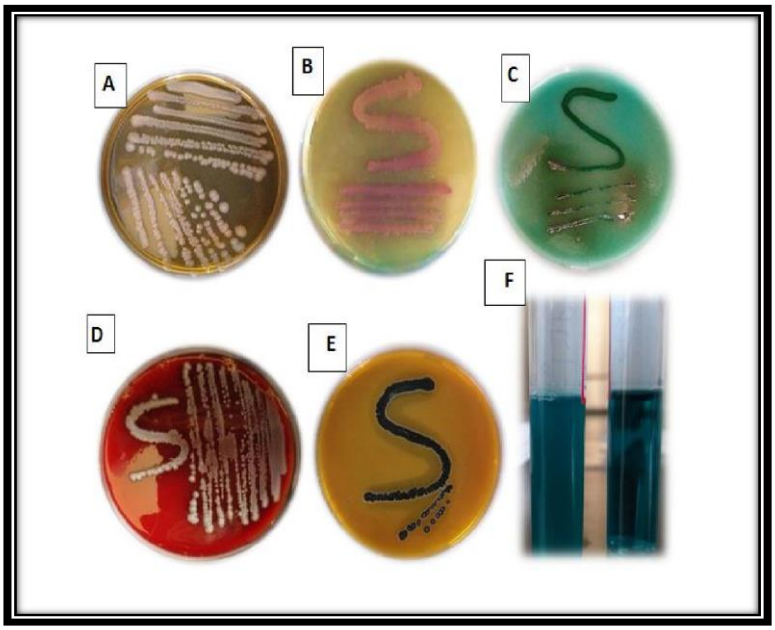


Figure 2. Gel electrophoresis image of polymerase chain reaction product of *ompX* gene of *Salmonella enterica* isolates,1.5% agarose.80 volts 40 min. Lane (1-30) amplified PCR product of *the ompX* gene. Isolates numbered (1-12) represent *Salmonella paratyphi* B, (13-22) represent *Salmonella typhimurium*, and (23-30) represent *Salmonella typhi*.

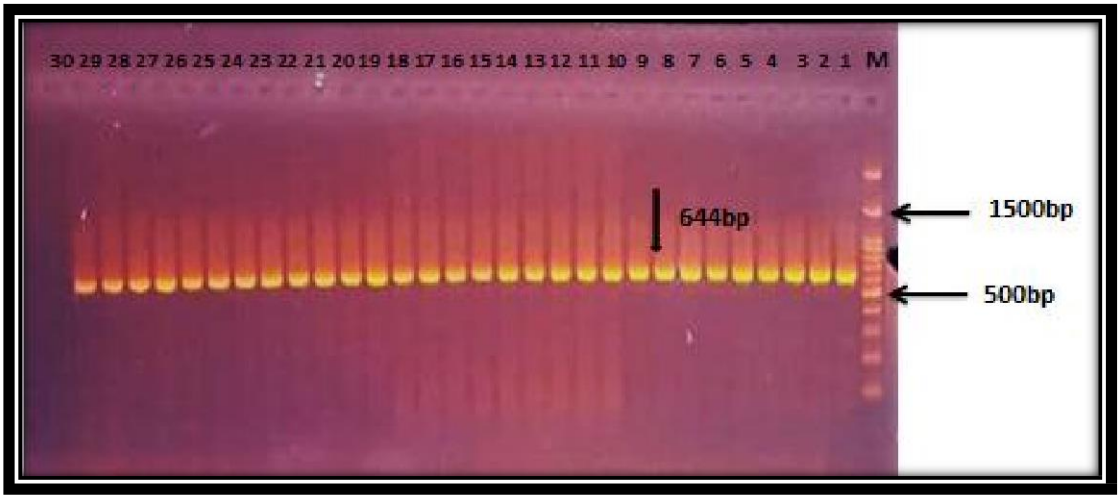
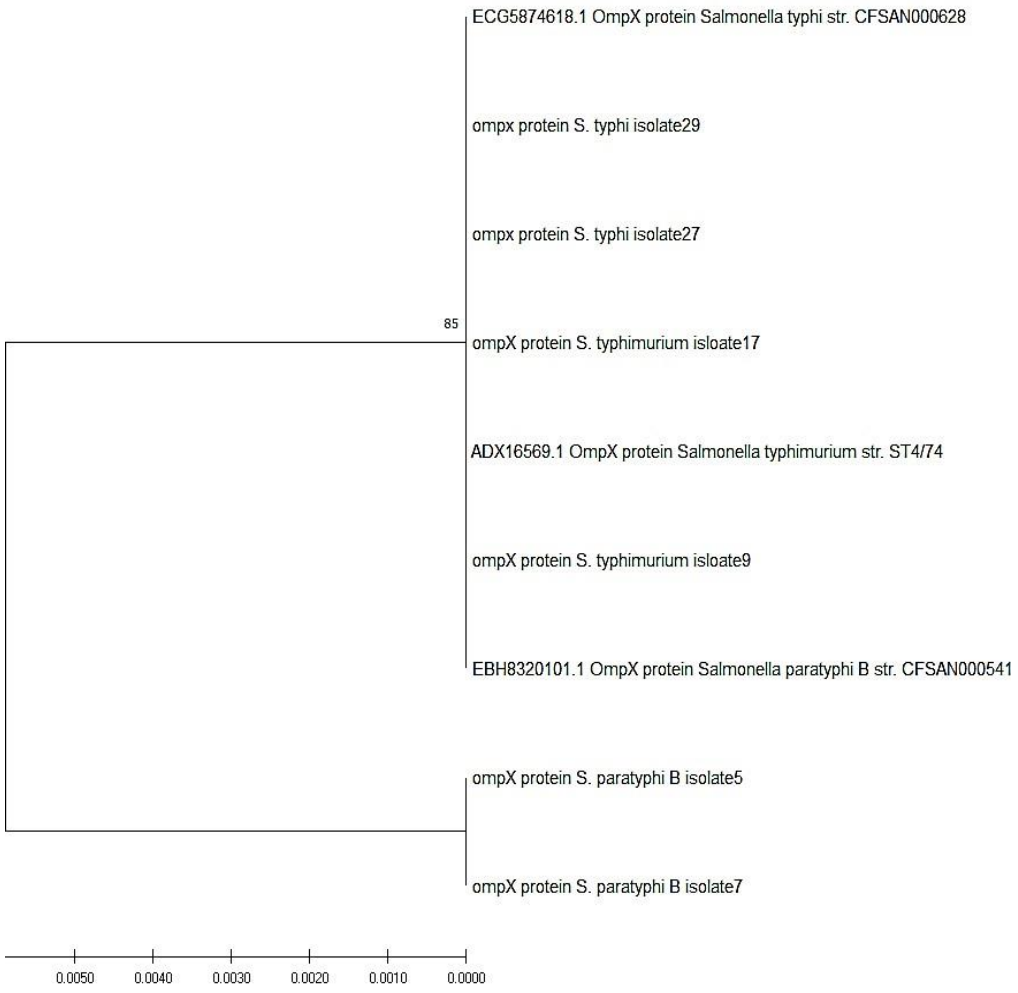


Figure 3. Phylogenetic tree of the ompX protein of *Salmonella enterica serovars* with standard *Salmonella enterica* form NCBI.



Discussion

An opportunistic pathogen frequently linked to outbreaks of foodborne illness is *Salmonella*. Because of its widespread circulation, public health is seriously threatened [12]. The primary pathogenic mechanisms of *Salmonella typhi* species are caused by virulence genes, which are expressed in response to physiological and environmental stimuli [13, 14]. Virulence factors can abuse hosts to increase a pathogen's fitness. Numerous genes encode virulence factors, which can be found on bacteriophages, virulence plasmids (pSLT), *Salmonella* pathogenicity islands (SPI), or at another position on the chromosome [15-17].

In the present study, all the isolates carried the *ompX* gene, and there was genetic variation of outer membrane protein in *Salmonella* species local isolates compared with global standard strains. According to post-transcriptional control, *ompX* was found to accumulate abnormally in a strain of *Salmonella* [18]. In bacteria, *ompX* has been demonstrated to play a role in both bacterial defense against the host's complement systems and penetration of host cells, neutralizing host defense mechanisms [19]. Cellular aggregation, adhesion to and internalization into host cells, serum resistance, and the *ompX* display in the OM were evaluated [20]. The overproduction of *ompX* was thought to be an adaptive response of the bacteria to environmental stressors [21]. By analogy, the pathophysiology of UPEC in kidney cells was discovered to be influenced by *ompX*, an outer membrane protein, which was studied in 2021 in

relation to virulence and flagellar expression in the uropathogenic *E.coli*. The *ompX* mutant remained attached to the epithelial cells at a degree comparable to that of the parent strain, despite the fact that bacterial internalization and assembly inside the kidney epithelial cells were decreased and colonization of the mouse urinary system was hindered upon *ompX* deletion [22].

Previous studies have reported a high prevalence of various virulence genes across different *Salmonella* serotypes. For instance, Radhi and Kadhim [23] demonstrated molecular detection of several virulence genes, including *ViaB, InvA*, and *Flic-d*, in *Salmonella typhi.* Also, the presence of these genes was revealed in the bacterial strains with a 100% detection rate. Additionally, genetic mutations of a non-conservative nature were observed in the amino acid sequences of certain bacterial strains. Yue *et al.* [24] assessed 61 *Salmonella* isolates that were taken from the feces of children who had severe diarrhea. They found that all of the isolates (100%) tested positive for the *prgH* gene, whereas 51 isolates (83.61%) tested positive for *sopB* and 11 isolates (18.03%) tested positive for *pefA*. The prevalence frequencies of the virulence genes *sipA, pefA*, and *spvC* were found to be 77.6%, 10.3%, and 1.9%, respectively, in another investigation by Qiao *et al*. [25]. He *et al*. [26] also pointed out that while *spvC* and *pefA* were detected at lower frequencies, the majority of virulence genes, such as *ssaR, sipA, sifA, sopE2, sopB, prgH*, and *stn*, showed a relatively high distribution. The virulence genes *lpfA, csgA, pagC, msgA, spiA, sitC, iroN, sipB, orgA, hilA, sopB, sifA, avrA,* and *sivH* were found in 14 out of 24 isolates from human cases of gastroenteritis (58.33%), according to Lozano-Villegas *et al*. [27-29]

Conclusion

The investigation of virulence genes within *Salmonella* strains plays a pivotal role in the identification of pathogenicity, comprehension of potential transfer mechanisms, and evaluation of the associated risks. Therefore, the development of an effective methodology for the detection of *Salmonella* virulence genes is imperative for the prevention and management of diseases.

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