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RESEARCH ARTICLE

Salmonella enterica Serovar Enteritidis and Typhymurium: Phenotypic, Molecular Detection and Sequencing of Quorum Sensing

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Abstract

Bacterial messaging and chatting, or Quorum sensing (QS) is a way that permitting the coordination behaviors of groups between many common bacterial pathogens. Objectives: Phenotypic detection of Quorum sensing production followed by Molecular Detection and Sequencing of its gene *sdiA*. Materials and Methods: A primers pair for PCR detection of *sdiA* gene of *Salmonella enterica* serovar enteritidis and typhymurium had been designed for its detection and followed by its sequencing for detection of mutation using automated sequencing. These were preceded by phenotypic detection of QS. Results: Quorum sensing produced by *S. enterica* was studied. Results revealed that homoserine lactone production causes the appearance of bacterial cells aggregation, which appears best after 4hours of incubation where it is representing a maximum concentration of homoserine lactone. In addition, *sdiA* gene is present in all isolates. Sequencing of *sdiA* gene from isolates of *Salmonella enterica* serovar enteritidis and typhymurium propose that there were 8 mutations in three isolates, also gave identity in a percentage of (98-99%) with standard strand according to NCBI web site.

Keywords: *Quorum sensing, Salmonella enterica, sdiA, sequencing.*

Introduction

Quorum sensing (QS) is a machinery apparatus that help bacteria in regulation of gene expression in association with cell density. In addition, it is a type of chemical communication between members of same species and nearby species, as it arises among many pathogenic bacterial types as a coordination behavior between groups; as control of virulence factor production, colonization of host cells and formation of biofilm at high densities of bacterial populations [1].

Auto inducers are compounds like hormones produced by bacteria that interacting with regulatory proteins after reaching a high concentration threshold. N-acyl L-homoserine lactone (AHL) with LuxR-type receptors of Gram-ve bacteria is is responsible for regulation of cell-cell signaling process [2]. *sdiA* found in many genera as Enterobacter, Klebsiella, Salmonella and Escherichia that responding to signals of AHL produced by further species and enhance genes regulation involved in host colonization [3]. A low, but constant signals of AHL are produced as a basal level; followed by a rapid diffusion into the local environment.

By the way of growing bacterial populations, the AHL concentration also increases till it reaches the intracellular threshold level, productive binding of AHL: LuxR type protein achieved that activating the genes transcriptions involved in various groups of behaviors [4]. Food borne pathogenic *S. enterica* serovar Typhimurium commonly contains *sdiA* gene that shows high sequence

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identity with same gene from other genera as, *S. Typhimurium*; thus, it is widely used as a target of many researches [5]. All these facts had been applied to control infections and severity of diseases, as a significant decrement in the expression of virulence factor can be obtained by inactivating the system of quorum sensing; since the expression of virulence genes among various pathogenic bacteria is centrally controlled by Quorum sensing, this providing a vital object for future strategies of controlling infectious bacterial diseases [6].

Salmonella spp. is one of the commonly known zoonotic foodborne pathogens for humans and animals and transmitting among them. In many nations, Salmonellae are the primary foodborne pathogene causing outbreaks of infections. Infections caused by *Salmonella enterica* still considered as a chief health problem worldwide, contributing to the economic load associated with surveillance, prevention and treatment of disease [7].

Materials and Methods

Collection of Samples

Samples Collection and Bacterial Isolation

This work included one hundred and fifty (150) diarrheic patients with age less than 10 years. Those patients were admitted at Babylon Teaching Hospital for Women and Children from April to September 2019. Watery stool samples (1gm) were collected in a sterile plain tube containing peptone water. These samples were applied for cultivation and isolation of S*almonella enterica* isolates, using XLD, S.S. and BGA agar media followed by its differentiation into serovar enteritidis and typhimurium serologically.

DNA Extraction and Molecular Detection of *sdiA* gene by Conventional PCR:

DNA extraction was done according to the genomic DNA purification kit supplemented by manufactured company (Geneaid, UK), in order to be used in PCR.

The primer sequences of *sdiA* gene (forward *sdi A1*: AATATCGCTTCGTACCAC and reverse *sdi A2*: GTAGGTAAACGAGGAGCAG) and PCR condition used for amplification (starting with initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30sec, annealing at 52ºC for 40sec and extension at 72ºC for 60sec, finally 72ºC for 7min as a final extension) [8]. PCR mixture was prepared by adding 12.5 µl of Green master mix, 2.5 µl templates DNA, 1.5 µl from forward primer and 1.5 µl from revers primer, final volume was completed to 25 µl by adding nuclease free water. The PCR amplification products were visualized by electrophoresis on 1% agarose ladder (promega, USA).

Phenotypic Detection of Quorum Sensing

Quorum sensing detection was done depending on the procedure explained by [9, 10]; where aspartic acid (1%) was added to Luria-Bertoni broth (LB), then subdivided into six flasks. Later on, incubation with Salmonella isolates, and the flasks were incubated at time intervals of [2, 3, 4, 5, 6, 24] hr at 37°C. At the end of these periods 0.01% KCN was applied with re-incubation for 18hr. followed by filtration of the media by 0.4mm Millipore filter; then the filtrated was dialyzed against KCN-free LB broth for 24hr. and use the supernatant for detection of quorum sensing.

Mixture of a drop of fresh bacterial growth with a drop of supernatant on a clean slide followed by gram staining and examining the slide under microscope. Bacterial cell aggregations indicate positive result. Homoserine lactone production was detected via separating the supernatant from culture media and dialyzed verses LB-free of KCN, then after 24 hrs media containing homoserine was inoculated with Salmonella isolates with 24 hrs incubation. Brands test used to detect homocystein or methionine synthesis via conversion of homoserine to homocysteine.

Detection of *sdiA* Gene by Automated Sequencing

After detection of PCR products, several obtained DNA amplicons were sent for sequencing process by Macro gene Company/ USA, in order to detect genes identity comparing it by blast program with the original genes in gene bank, which is available at the national center biotechnology information (NCBI).

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Results

Detection of Quorum Sensing Phenotypic

After the whole time of this work, results gave (15) *Salmonella enterica* isolates; (10) of them were serovar enteritidis and the remaining (5) were serovar typhymurium. During the phenotypic detection of QS production among all *Salmonella enterica* isolates, in culture medium and after the addition of KCN, there would be accumulation of homoserine; this occur as the KCN inhibit synthesis of threonine synthase inhibition. Brands test was also done to be sure about homoserine lactone production that indicates quorum sensing formation, as the positive result appeared as agglutination, Figure (1). These results were checked at intervals with a best and maximum amount after 4 hours.

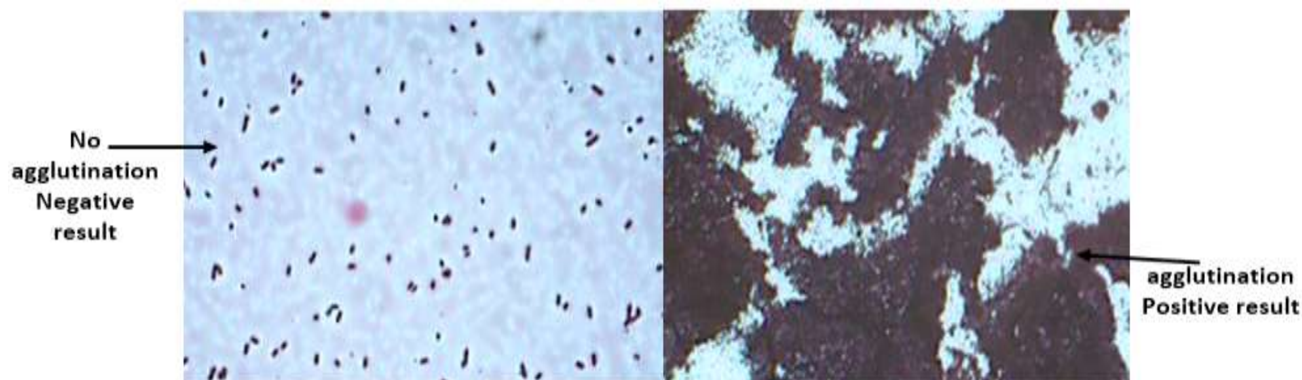

Figure 1: Detection of quorum sensing in *Salmonella enterica* (100x) left: control (absence of homoserine); right: positive result

Molecular Detection of *sdiA* gene by Conventional PCR

Conventional PCR procedure was performed for detection of *sdiA* gene; it was done via application of two oilgonucleotide DNA fragments acting as specialized primers for virulence gene of *S. enterica*. Results shown that *sdiA* gene was obtained positive for all isolates with an amplicon size at about 274bp, Figure (2).

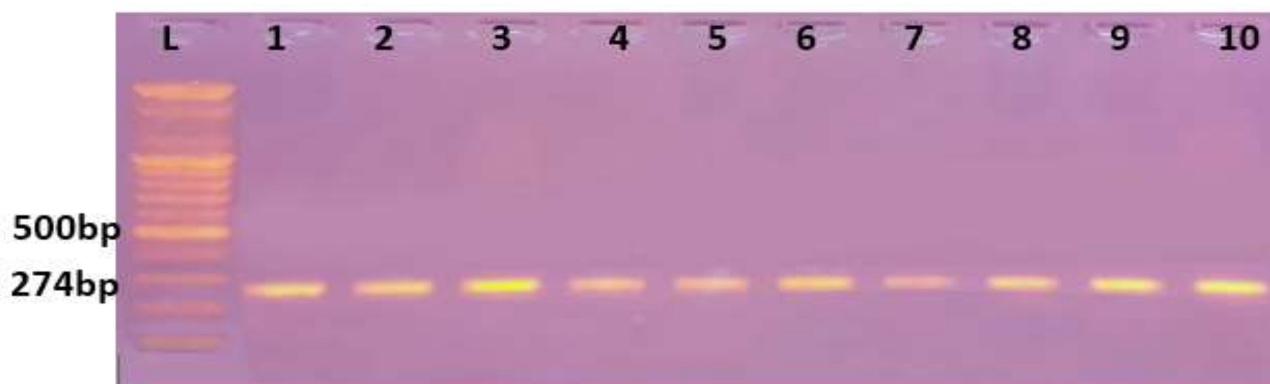

Figure 2: Detection of *sdiA* gene PCR products by agarose gel electrophoresis (1%) at 70 volt for 30 min with U.V light visualization at 280 nm and ethidium bromide staining. L: 1500 bp ladder; positive gene results at lane (1-10) with size of product is 274 bp for *sdiA* gene

The Nucleotides Sequence of *sdiA* Genes

The data of the sequence of nitrogenous bases of the outputs of the PCR reaction for 3 samples of each gene from the *sdiA* gene after sending 50 mL of the output of PCR for each sample with the primers of each gene to Macrogen company in the United States by Genetics company for products and services bio-medical technology Amman Jordan. All results were compared with the sequences of global strains registered from different parts of the world by a computer program that is Mega 6 results were compared with the original sequence of each gene.

Sequence Analysis of *sdiA* gene

Sequence analysis *sdiA* gene revealed that there is some variation of identity is (98-99%) whene compared with standard isolates as showen in the Figuer (3) and Table (1) Summarized that (8) mutations were detected in (3) of samples of the gene *sdiA*,

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where more than one mutation were identified in each sample, as the type and location of each mutation could lead to the differences in the effect of these mutations. Some of the obtained mutations leading to genetic code changes; and then amino acids changes at the translation level.

Isolate 1:

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Salmonella enterica subsp. enterica strain NCTC9684 genome assembly, chromosome: 1

Sequence ID: LR134233.1 Length: 4610917 Number of Matches: 1

Range 1: 1903784 to 1904018 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
411 bits(222)	9e-111	232/236(98%)	3/236(1%)	Plus/Plus

```
Query  11       CGCATTACCAGTCCG-ATACTATTTTGCGATCGATCCGGTATTAAAGCCGGAAAATTTCA  69
Sbjct  1903784  CGCATTACCAGTCCGAAAACTATTTTGCGATCGATCCGGTATTAAAGCCGGAAAATTTCA  1903843
Query  70       GGCAGGGTCATTTACATTGGGATGACGTGCTATTTCATGAAGCGCAGGCGATGTGGGATG  129
Sbjct  1903844  GGCAGGGTCATTTACATTGGGATGACGTGCTATTTCATGAAGCGCAGGCGATGTGGGATG  1903903
Query  130      CCGCCCAGCGTTTCGGATTACGCAGAGGCGTAACCCAGTGTGTGATGTTGCCGAACCGGG  189
Sbjct  1903904  CCGCCCAGCGTTTCGGATTACGCAGAGGCGTAACCCAGTGTGTGATGTTGCCGAACCGGG  1903963
Query  190      CGCTGGGCTTTTTATCTTTCTCCCGTAGCAGTTTACGCTGCTCC-CGATTTACCTA  244
Sbjct  1903964  CGCTGGGCTTTTTATCTTTCTCCCGTAGCAGTTTACGCTGCTCCTCG-TTTACCTA  1904018
```

Isolate 2:

Download GenBank Graphics

Salmonella enterica subsp. enterica serovar Typhimurium strain ATCC 14028 chromosome, complete genome

Sequence ID: CP034230.1 Length: 4869644 Number of Matches: 1

Range 1: 1994331 to 1994566 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
412 bits(223)	2e-111	233/237(98%)	3/237(1%)	Plus/Minus

```
Query  12       CGCATTACCAGTCCGA-TACTATTTCGCGATCGATCCGGTATTAAAGCCGGAAAATTTCA  70
Sbjct  1994566  CGCATTACCAGTCCGAAAACTATTTCGCGATCGATCCGGTATTAAAGCCGGAAAATTTCA  1994507
Query  71       GGCAGGGTCATTTACATTGGGATGACGTGCTATTTCATGAAGCGAAGGCGATGTGGGATG  130
Sbjct  1994506  GGCAGGGTCATTTACATTGGGATGACGTGCTATTTCATGAAGCGAAGGCGATGTGGGATG  1994447
Query  131      CCGCCCAGCGTTTCGGATTACGCAGAGGCGTAACCCAGTGTGTGATGTTGCCGAACCGGG  190
Sbjct  1994446  CCGCCCAGCGTTTCGGATTACGCAGAGGCGTAACCCAGTGTGTGATGTTGCCGAACCGGG  1994387
Query  191      CGCTGGGCTTTTTATCTTTCTCCCGTAGCAGTTTACGCTGCTCC-CGATTTACCTAC  246
Sbjct  1994386  CGCTGGGCTTTTTATCTTTCTCCCGTAGCAGTTTACGCTGCTCCTCG-TTTACCTAC  1994331
```

Isolate 3:

Download GenBank Graphics

Salmonella enterica subsp. enterica serovar Typhimurium strain ATCC 14028 chromosome, complete genome

Sequence ID: CP034230.1 Length: 4869644 Number of Matches: 1

Range 1: 1994331 to 1994566 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
424 bits(229)	1e-114	234/236(99%)	2/236(0%)	Plus/Minus

```
Query  17       CGCATTACCAGTCCGAA--CTATTTCGCGATCGATCCGGTATTAAAGCCGGAAAATTTCA  74
Sbjct  1994566  CGCATTACCAGTCCGAAAACTATTTCGCGATCGATCCGGTATTAAAGCCGGAAAATTTCA  1994507
Query  75       GGCAGGGTCATTTACATTGGGATGACGTGCTATTTCATGAAGCGAAGGCGATGTGGGATG  134
Sbjct  1994506  GGCAGGGTCATTTACATTGGGATGACGTGCTATTTCATGAAGCGAAGGCGATGTGGGATG  1994447
Query  135      CCGCCCAGCGTTTCGGATTACGCAGAGGCGTAACCCAGTGTGTGATGTTGCCGAACCGGG  194
Sbjct  1994446  CCGCCCAGCGTTTCGGATTACGCAGAGGCGTAACCCAGTGTGTGATGTTGCCGAACCGGG  1994387
Query  195      CGCTGGGCTTTTTATCTTTCTCCCGTAGCAGTTTACGCTGCTCCTCGTTTACCTAC  250
Sbjct  1994386  CGCTGGGCTTTTTATCTTTCTCCCGTAGCAGTTTACGCTGCTCCTCGTTTACCTAC  1994331
```

Figure 3: sequencing of *sdiA* gene in three isolates

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Table 1: Types of mutations in the *sdiA* gene sequence in *S. enterica* bacteria

No. of sample	Wild type	Mutant type	Site	Change in amino acid	Type of mutation	Effect
Salmonella entritidis (1)	AAA	-AT	1903799 1903801	Deletion A	Deletion	Frame shift
	CCT	CC-	1904007	Deletion T	Deletion	Frame shift
	CGT	CGAG	1904010/1904011	Insertion A	Insertion	Frame shift
Salmonella typhimrum (2)	AAA	A-T	1994550 1994549	Deletion A	Deletion	Frame shift
	CCT	CC-	1994342	Deletion T	Deletion	Frame shift
	GCT	GCAT	1994340/1994341	Insertion A	Insertion	Frame shift
Salmonella typhimrum (3)	AAA	AA-	1994549	Deletion A	Deletion	Frame shift
	ACT	-CT	1994548	Deletion A	Deletion	Frame shift

Determine the type of Mutations and Percentage

The genetic structure of *sdiA* gene analyzed by sequencing revealed that there are genetic changes, and the data shown in the Table (2) that there are 6(75%) Deletion mutations and 2 (25%) Insertion mutations.

Table 2: types of mutations in *sdiA* gene and percentage

Type of Mutations	Numbers	Percentage
Deletion	6	75%
Insertion	2	25%
Total	8	100%

Mutation Effects

sdiA gene mutations could create changes in the gene organization with changes in its activity. The result show there are 8 (100%) of Frame shift mutations that leading to reading shift leading to a totally diverse type of translation originally,with big changes in the translated protein.

Discussion

Ingestion of water or food sources contaminated with urinary or fecal excreta of animals acting as reservoirs of Salmonella especially *Salmonella enterica*, can cause gastro-intestinal problem that is called salmonellosis [11]. It is linked with very serious mortality and morbidity, also it is a sever public health problem around the world [12]. Many studies recorded various reports regarding the appearance of salmonellosis cases among diarrheic patients especially children; as Al-Jobouri and his colleagues [13] revealed that *S. enterica* was recorded at about 70% of cases in (1-14) years age group.

Also, Al-karawiy, [14] had detected Salmonella cases at (10%) from diarrheal patients of children group in Al-Qadsia hospitals. While, Al-Janabi [15] at Al-Qadsia province, was studied Salmonellosis in (608) diarrhea cases among children and found proportion of isolates as (14.47%).This increment in the susceptibility of children Salmonellal to infection could be due to young age, malnutrition, lack of breast feeding and immune deficiency.

Salmonella spp. have multiple regulatory and virulence genes that maintain their growth and multiplication within the host [16].

Salmonella enterica has abilities to employ multiple and various virulence factors produced at different stages of pathogenesis, in order to begin successful infection course. Most of *S. enterica* virulence genes involving in intracellular pathogenesis and host cell invasion are chromosomally located mainly on pathogenicity islands, that best to be detected via PCR technique [17].

In present study, *sdiA* gene was detected in all isolates, that is related to the results obtained by [8, 18] who were found that *sdiA* gene was identified at highest frequencies reaching 100%., and this high availability of this gene could help to use it as a PCR target for detection of Salmonella spp.; where sdiA is (Suppress division inhibitors A), which responsible for *rck* gene regulation, that are important in bacterial adhesion, invasion of epithelial cells with their resistance to complement [19].

Salmonella SdiA as a signaling mechanism that controlling variable pathways dealing with expressing a number of virulence factors; like its role in the regulation of many accessory factors central in bacterial colonization and survival in the intestine [20]. Quorum sensing has many important roles in cell physiology with chromosomal replication inhibition, use quorum sensing to regulate division, based on availability of

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nutrients, competition from other microbes, and assessment of population density [21]. Products of the gene amplification showed wide variations of nucleotides which confirmed the gene polymorphism. Isolates alignment revealed that *sdiA* gene showing little conservation, but these mutations can alter gene functions. However, it was documented that the mutation in the sequences of the genes that encode them including insertion, deletion or integration of forign DNA between isolates effect on the sequence composition [22].

Molecular bacterial evolution can be easily understood by High-through put sequencing and promise to lighten the in vivo dynamics of bacterial infection and carriage. The role of genetics, circus stance and chance in invasive bacterial disease is let to be determined, but the comprehensive characterization of bacterial genetic version in side the host cells is an important step.

Amino acid sequence truncation by loss- of function mutation has an essential role in pathogenesis, as the point mutation of this type could be quickly affect radical functional changes, many mutation as there have no effect on the ability of the protein to work or result in a loss of function. When this occur the bacteria have these mutations are less suited to survive.

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