Research Article

Use of Quorum Sensing Gene *Sdi*A as a Molecular Marker for *Salmonella* Diagnosis

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Abstract | The alterations in cell density of the bacteria as a result of the gene expression mechanism is called quorum sensing and these changes in *Salmonella* has been an essential issue for a long time. The quorum sensing gene (*sdiA*) found in *Salmonella spp*. is an important regulatory gene for *Salmonella* survival, colonization and communication with other bacteria and hosts. *Salmonella* live in the human intestine, which harbors a great density and variety of bacterial cells, in addition to the other flora exist in the colon and all of them communicate amongst themselves and with the host itself to make a change for adaptive processes, such as antibiotic production, invasion of host cells and biofilm formation . Twenty-eight local Egyptian *Salmonella* isolates from different localities and different sources in Egypt such as, human stool, Egyptian cattle egrets and storks and grilled chicken from electric grills, were tested for the presence of *sdiA* gene by using PCR and compared with four non-*Salmonella* local isolates. All *Salmonella* isolates were PCR-positive for the *sdiA* gene (274-bp product). All non-*Salmonella* isolates were PCR-negative for the *sdiA* gene. Sequencing of *sdiA* gene revealed thet there were more than 99 % similarity to *sdi*A gene sequences existing in the GenBank database of different serotypes of *Salmonella enterica* strains. Therefore, it can be suggested that the *Sdi*A gene is conserved among *Salmonella enterica* strains regardless of their serotypes. This work provide evidence that the *sdiA* gene is necessary for *Salmonella* virulence and the (*sdiA*) PCR assay is a unique, highly specific molecular marker for the diagnosis and detection of *Salmonella*.

Keywords | *Salmonella*, Virulence, Quorum sensing, PCR, Sequencing

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INTRODUCTION

Salmonella spp. are gram-negative bacilli spread among domestic and wild animals and humans that cause severe illnesses, such as diarrhoea, fever and general fatigue (Baired-Parker, 1990). *Salmonella* spp. are a major food-borne pathogen in animals and humans (Humphery, 2002). Hence, rapid detection of *Salmonella* in clinical samples, foodstuffs and water is critical for prompt and accurate diagnosis of *Salmonella*, which in turn helps in control and prevention of the disease. Molecular diagnosis by using PCR assay is more accurate and reliable technique for the detection of pathogens. Interestingly, several PCR techniques have been developed for detection of numerous genes in *Salmonella,* for example (Rahn et al., 1992), *agf*A (Doran et al., 1993), *hil*A and *sir*A (Guo et al., 2000) 16S rRNA (Iida et al., 1993), *via*B (Hashimoto et al., 1995), and *ttr* (Malorny et al., 2004). However, the *inv*A gene is the most commonly used for the diagnosis of *Salmonella* due to it has unique DNA sequences (Hara-Kudo et al., 2005; Abdel-Aziz, 2016). The majority of *Salmonella spp*., except *S. litchfield* and *S. Senftenberg,* are found to harbour *inv*A gene. In the invasion-associated protein secretion mechanism, the *invA* gene encodes an essential

compound (Gala'n et al., 1992). Besides, the *inv*A gene has been found to be located in region that constitutes a pathogenicity island –PAI-, and it is in some *Salmonella* serotypes unstable, such as *S. litchfield* and *S. Senftenberg* (Ginocchio et al., 1997).

Although, in the past, many researches have revealed that most of the bacteria use several quorum-sensing systems, but the biological significance of this phenomenon remains unknown. Bacterial cells are able to modify the gene expression pathway in response to extracellular signals. In contrast, certain bacteria that can release and detect signalling compounds to control gene expression using quorum sensing were first detected in marine *Vibrio fischeri* (Nealson and Hasting, 1979). Quorum sensing is the communication phenomenon between bacteria, and it controls cellular aggregation-dependent factors, such as antibiotic production, invasion of host cells and biofilm formation (Wei and Zhao, 2018). The bacteria secrete acyl homoserine lactone (AHL), which is a species-specific autoinducer-1 (AI-1). AI-1 is an extracellular signalling molecule that is responsible for the activation of quorum sensing (Zhang and Li, 2016). Gene transcription, which is regulated by the regulatory gene *sdiA* (LuxR family), is induced as soon as the threshold level is reached.. The *sdi*A gene of *Salmonella* (LuxR family) regulates the intestinal survival, colonization and invasion (pathogenicity) of *Salmonella* (Ahmer, 2004). Host entrance and adherence to intestinal mucosa are important for *Salmonella* pathogenicity in cell invasion. There are interacting signals between *Salmonella* and the host. These signals lead to changes in ruffling of the membrane and the entry of *Salmonella* (Gala'n, 1995). Therefore, the development of a molecular marker for *Salmonella* spp. detection is very important.

The aim of the current study was to examine the possibility of using the *sdi*A gene as a molecular marker for diagnosis of local Egyptian *Salmonella* strains recovered from different sources, including human stool, foodstuffs, and carriers gathered from the Cairo, Giza and Menofeia governorates. Moreover, sequencing of the *sdi*A gene was used to detect the epidemiological link between the different Egyptian *Salmonella* isolates.

MATERIAL AND METHODS

Bacterial isolates

Twenty-eight local Egyptian *Salmonella* isolates from the Cairo, Giza and Menoufia governorates were collected from different sources, such as 75 human stool from clinically affected persons tested by the Widal test, 35 Egyptian stork (major *Salmonella* carriers) and 30 from multilayer electric grills, grilled chicken. All strains of *Salmonella* were characterized culturally, biochemically, serologically and molecularly by using a PCR assay for the *invA* gene as previously studied by (Abdelmalek et al., 2019), as it is the most common PCR assay for *Salmonella* isolate identification,. Four Local non-*Salmonella* bacterial isolates were used as negative controls for the *sdi*A gene as follows: *E. coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*. They were local isolates and were identified culturally, biochemically and molecularly.

The extraction of DNA

On nutrient agar medium (SIGMA), the local *Salmonella* isolates were grown, and the bacterial cells were harvested from the culture plates using a sterile cotton swab placed in an Eppendorf tube containing 1X PBSS. [1X phosphate buffered saline prepared by dissolving one tablet in 100 ml of distilled water (SIGMA)], and mixed well. The specimens were then centrifuged for 5 min at 9000 x g (washing was repeated three times with PBS) and then washed once with sterile distilled deionized water. Then, at 9000 x g for 5 min, centrifugation was performed and the supernatant was extracted. Subsequently, 100 µl of sterile deionized distilled water was suspended in the bacterial pellet. The samples were put in a heat block (Grant-Bio, England) for 5 min at 100OC and then cooled in ice water immediately. The specimen had been centrifuged, (Wang et al., 1996).

PCR amplification and electrophoresis

In a 25 µl reaction mixture containing 12.5- µl of 2X master mix - (EmeraldAmpGT, A2201-1, TAKARA, Japan), 0.5 µl (25 pmol) of each primer (*sdiA* F and R), 2 - µl of the extracted DNA and 9.5 - µl of free water from nuclease (Thermo Science, # R0581, USA), PCR amplification was performed.

The cyclic program consisted of denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 0.5 min. Then, final extension at 72°C for 7 min was carried out (Halasti et al., 2006). In electrophoresis using 1.5 percent (w/v) agarose gels (SERVA, Russia), all reaction products in 1X TAE running buffer (SERVA, Russia) were analyzed at 100 V for 30 minuts and imaged using a Spectroline UV transilluminator (Slimline Series, USA).

DNA sequencing

The amplified fragments of *sdiA* gene in the five selected *Salmonella* isolates that randomly selected from grilled chicken, human and stork in the same locality were sent to be sequenced at the Animal Health Research Institute (AHRI) in Dokki- El Giza . Using the GeneJET PCR Purification Kit (Thermo Scientific), the PCR products were purified and a DNA sequencer was used to make the sequencing. The Large Dye Terminator (V3.1 Cycle) Sequencing Kit was used to perform the sequencing step,

(Applied Biosystems). The gene sequences have been submitted in the National Center for Biotechnology Information (NCBI) GenBank database by using BankIT tool under the accession numbers MF942870, MF942871, MF942872, MF942873, MF942874 for the human stool, stork and grilled chicken-derived sequences, respectively.

The sequences obtained were determined by a BLAST analysis with GenBank (NCBI). To determine the relationship of our gene sequences recovered from various sources, phylogenetic tree analysis was used and related *sdi*A gene sequences obtained from the GenBank database. For Clustral W- multiple alignment, BioEdit software was used, and MEGA7 software was used using the maximum likelihood approach for the construction of phylogeny.

RESULTS

PCR RESULTS

All local Egyptian *Salmonella* isolates were PCR-positive for the *sdi*A quorum sensing gene (274-bp PCR product). The local non-*Salmonella* isolates *E. coli, K. pneumoniae, P. vulgaris* and *P. aeruginosa* were PCR-negative for the *sdi*A gene (Figure 1).

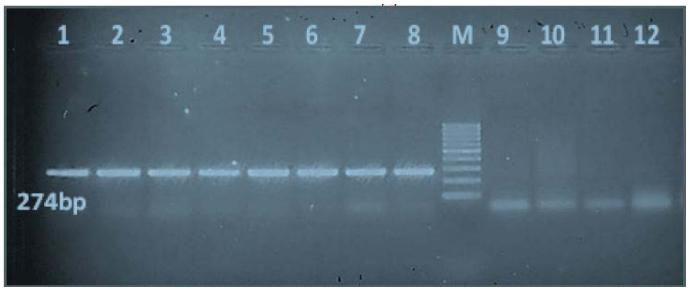

Figure 1: PCR results of the quorum sensing *sdi*A gene of local Egyptian Salmonella isolates in comparison with non-Salmonella isolates: Lanes 1-8: Salmonella isolates that were PCR-positive for the *sdi*A gene, which produces a 274-bp product. Lane M: 100-bp molecular ladder. Lanes 9-12: Negative PCR results for the *sdi*A gene of four non-Salmonella isolates (*E. coli, Klebsiella pneumonia, Proteus vulgaris, and Pseudomonas aeruginosa*).

Table 1: Oligonucleotide sequence of the quorum sensing *sdi*A gene.

Gene	Sequence	Product	Ref.
*sdi*A F:	5-AAT ATC GCT TCG TAC CAC-3	274 bp	(Halatsi *et al.*, 2006)
*sdi*A R:	5-GTA GGT AAA CGA GGA GCA G-3		

SEQUENCING RESULTS

In this analysis, the representative sequences were deposited under accession numbers MF942870, MF942871, MF942872, MF942873, and MF942874 for the human stool, stork and grilled chicken-derived sequences, respectively into the GenBank database. The *sdi*A gene nucleotide sequence data available in GenBank were selected based on the criteria of the available information regarding the source, location and year of isolation (Table 2). The five amplified *sdiA* gene PCR products were sequenced and matched with the other associated GenBank NCBI-BLAST gene sequences of *sdiA*. The ≤ 99% similarity to the *sdi*A gene of *Salmonella enterica* strains showed that the primers included here exclusively amplifies the 274 bp fragment of the target gene. The phylogenetic analysis results of 5 *Salmonella enterica* isolates based on *sdi*A gene sequences are shown in Figure 2. Initially, the *sdiA* gene sequences of these isolates were compared to those published in GenBank for *Salmonella* enterica strains (Table 2).

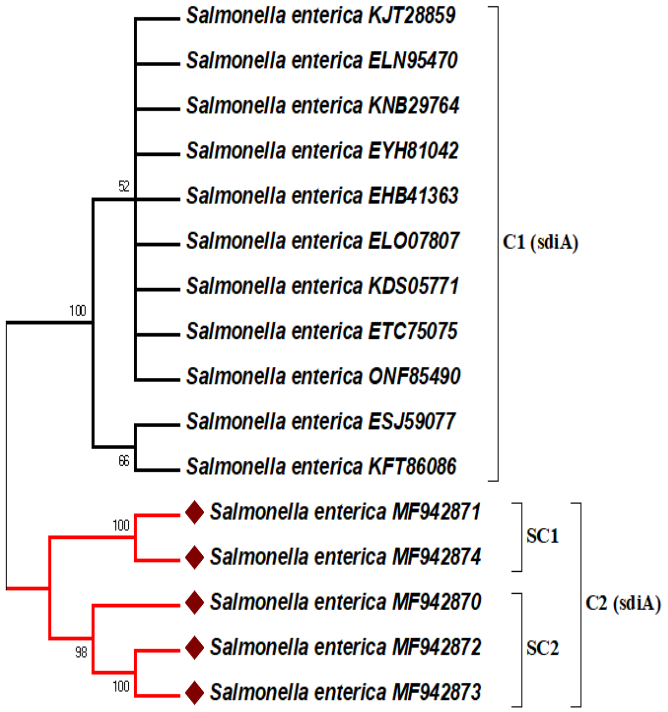

Figure 2: Maximum likelihood phylogenetic tree based on *sdi*A gene sequences showing the relationship between our study sequences and 11 representatives of sequences retrieved from GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Only branches with bootstraps greater than 50 are labelled. The bacterial isolate sequenced in this study is indicated by a red diamond.

DISCUSSION

Salmonella is a very significant food-borne pathogen that causes many public health problems. Prompt and accurate diagnosis is crucial for the detection of *Salmonella* spp. Molecular diagnosis is more reliable and accurate for pathogen diagnosis than other methods.

Table 2: Isolation source, location and accession no. of *Salmonella* spp.

Sample no.	Serotype	Isolation source	Location	Accession no.
1	*Salmonella* Heidelberg	Environmental sample	USA	KJT28859
2	*Salmonella* Enteritidis	Human	USA	ELN95470
3	*Salmonella* Infantis	Food sample	Portugal	KNB29764
4	*Salmonella* Heidelberg	Chicken breast	USA	EYH81042
5	*Salmonella* Infantis	Human	USA	EHB41363
6	*Salmonella* Enteritidis	Chicken breast	USA	ELO07807
7	*Salmonella* Heidelberg	Human	USA	KDS05771
8	Salmonella enteric	Bostourus (faecal sample)	USA	ETC75075
9	*Salmonella* Typhimurium	Chicken meat	Brazil	ONF85490
10	Salmonella enteric	Dry milk	London	ESJ59077
11	*Salmonella* Bareilly	Food sample	USA	KFT86086
12*	*Salmonella* Typhimurium	Human stool	Egypt	MF942870
13*	*Salmonella* Typhimurium	Stork (carrier)	Egypt	MF942871
14*	*Salmonella* Typhimurium	Grilled chicken	Egypt	MF942872
15*	*Salmonella* Typhimurium	Grilled chicken	Egypt	MF942873
16*	*Salmonella* Typhimurium	Grilled chicken	Egypt	MF942874

*Gene sequences enrolled in our study.

Many *Salmonella* studies have been focused on the *invA* gene in different sources; including, animals and humans from different serotypes (Herbert and Hensel, 2004).

Recently, quorum sensing is an essential regulator of bacterial pathogenicity, particularly in *Salmonella* (Gala'n, 1995; Halasti et al., 2006). The detailed mechanism is still unknown. The *sdi*A gene is a type of *Salmonella* regulatory receptor (LuxR family) that is present in the centrosome 42 region (far from the centrosome 63 region of the *invA* gene) and it does not regulate the virulence-associated secretion mechanism but does regulate essential factors that control intestinal survival and colonization. Until now, the *sdi*A gene has been shown to initiate genes that help only one of the *Salmonella* bacteria adhere to the intestinal mucosa (Ahmer, 2004). In the present study, the *sdi*A gene was examined as a molecular marker for diagnosis of local Egyptian *Salmonella* isolates from different sources, and compared with non-*Salmonella* local isolates *E. coli, K. pneumoniae, P. vulgaris* and *P. aeruginosa* (Figure 1). Based on the aforementioned data, this study proved that the *sdi*A gene is highly specific for *Salmonella* spp. and could be used as a molecular marker for *Salmonella* diagnosis (Halasti et al., 2006).

DNA sequencing was accomplished to confirm that *sdi*A gene is a conserved gene in *salmonella* spp., and predict the epidemiological relationships between our study sequences and related *sdi*A gene sequences from the available GenBank NCBI-BLAST database. Among the study sequences, there was more than 99 percent matches to *sdi*A gene sequences of different serotypes of *Salmonella enterica* strains existing in the GenBank database. These results suggest that the *Sdi*A gene is conserved among *Salmonella enterica* strains regardless of their serotypes. Previous studies supported this suggestion by sequencing and analysing phylogenetic trees based on the *sdi*A gene (Herbert and Hensel, 2004; Campos-Galvão et al., 2015). Phylogenetic analysis of the amino acid sequences revealed the existence of two major clusters (C1 and C2) according to the geographical isolation with high bootstrap support value. These geographical clusters may be due to the *sdi*A gene partial sequence data and are scarce in Africa and the Middle East. Therefore, our study sequence isolates from Egypt with accession numbers MF942870, MF942871, MF942872, MF942873, and MF942874 are located on the same cluster (C2) and show no homology with other *sdi*A-related sequences (Figure 2). The majority of the *sdi*A gene data available in GenBank are from the USA and Latin America (Table 2). Egyptian isolates with the same serotype (*Salmonella typhimurium*) were divided into 2 subclusters (SC1 and SC2) with high bootstrap support values. SC1 shows that the study sequences of stork (wild bird) **(**MF942871) and the grilled chicken (MF942874) are highly similar to each other, which reflects the potential role of wild birds in the indirect transmission of *Salmonella* to humans through foodstuffs. This scenario spotlights on the public health implication of the distribution of wild birds, for example, storks, near restaurants in Egypt. Epidemiologically, wild birds that are attracted to human sewage outfalls may disseminate *Salmonella* to susceptible individuals through faecal shedding and shared environments and via direct contact (Afema and Sischo, 2016; Ahmed et al., 2019). In addition, we cannot deny the role of infected humans in the contamination of ready to eat food, and similar strains of *Salmonella typhimurium* were

found in human stool (MF942870) and grilled chicken (MF942872, MF942873), as shown in SC2 (Figure 2). This finding confirmed that cooks or food handlers who do not follow proper personal hygiene procedures may act as potential sources of recontamination of food after cooking.

Table 3: the different serotypes of the *salmonella* isolates.

Isolates/ numbers	Serotypes	No. (%)
Human/ 7	*S.* Typhimurium	3 (42.8)
	S. Enteritidis	1 (14.3)
	S. Virchow	1 (14.3)
	S. Haifa	1 (14.3)
	S. Kentucky	1 (14.3)
carriers stroke/ 10	*S.* Typhimurium	10 (100)
grilled chicken/ 11	*S.* Typhimurium	10 (100)

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AUTHOR'S CONTRIBUTION

Shaymaa Abdelmalek, Esraa Abdulmaged.Elshafiee, Wafy Hamed, Mona Kadry contributed to the collection of samples, isolation of strains, performing the molecular detection of target genes, analysis and interpretation of the data as well as writing the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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