

## Molecular Characterization of Some Virulence Genes of *Salmonella enterica* Serotype Sandow and Saintpaul Isolated from Environment of Dairy Farms at Assiut Province, Egypt

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### Abstract

Dairy farm environment could become contaminated with *Salmonella* following outbreaks of illness, colonization of animals or by general contamination. The present study investigated a total number of 95 samples collected from 3 dairy farms in 3 different localities at Assiut Province including air (25), water (35) and manure (35) samples for detection of *Salmonella* species by bacteriological isolation, morphological, biochemical and serological identification followed by molecular characterization for the presence of 6 virulence genes; *pefA* (700 bp), *mgcC* (677 bp), *stn* (617 bp), *sopB* (517 bp), *invA* (284 bp) and *avrA* (422 bp) in *Salmonella* isolates. Bacteriological examination revealed the isolation of 3 (3.2%) *Salmonella* isolates from totally examined samples, which were identified serologically as 1 (2.9%) *Salmonella enterica* serotype Sandow isolated from water and 2 *Salmonella enterica* serotype Saintpaul from air and manure (one from each with 2.9% of each). Molecular characterization of the 3 *Salmonella* isolates revealed that all 6 tested virulence genes; except *sopB* (517 bp); presented in *Salmonella enterica* serotype Sandow isolated from water, while all 6 tested virulence genes found in *Salmonella enterica* serotype Saintpaul isolated from manure and found that *invA* (284 bp) was the only virulence gene located in *Salmonella enterica* serotype Saintpaul isolated from air. This study highlighted on the potential sources for *Salmonella* contamination moreover the epidemiology of salmonellosis in dairy farms that necessitate following of strict healthful measures to reduce the risk of *Salmonella* infection that still constitutes a significant world zoonosis particularly through contamination of dairy farm environments that contribute to extend the recycling of *Salmonella* that considered the most vital sources of animal and human infection with *Salmonella*.

**Keywords:** *Salmonella*; Dairy; Environment; PCR; Virulence genes

### Introduction

As *Salmonella* colonizes the gastrointestinal tract, the organisms are excreted in feces which may be transmitted by insects and other animals to a large number of places and are generally found in polluted water. Salmonellae do not originate in water; therefore their presence denotes fecal contamination [1,2]. Human and animals that consume polluted water may shed the bacteria through fecal matter continuing the cycle of contamination.

*Salmonella* serovars are resilient microorganisms with a complex genomic system that makes the organism able to react to different harsh environmental conditions at the farm. Different stress factors that the bacteria may be exposed to include temperature, pH, osmotic shifts, and low beyond their normal growth range. More researches are required to understand why few *Salmonella* serovars are responsible for a majority of human diseases and demonstrate such unique reservoirs and pathogenesis. With a better understanding of serovars, mitigation methods can be implemented to control Salmonellae pre-harvest and postharvest levels [3,4].

Multiplication of *Salmonella* has been reported during storage of slurry but, in general, their numbers are reduced. *Salmonella* may survive for long periods in infected faeces, where their survival is depending on many factors, especially climatic conditions. S. Dublin

survived for at least 72 days in faeces on pasture during winter and 119 days in summer [5]. In moist, un-heaped faeces, survival up to 3-4 months has been reported.

*Salmonella* infections are an important cause of mortality and morbidity in cattle and sub-clinically infected cattle are frequently found. Cattle thus constitute an important reservoir for human infections [6-9].

*Salmonella* spreads around the farm environment on boots, tractors, other equipment, and surface water, effluent from animal accommodation, birds, rodents, and domestic animals. Every effort should be made to attain a very high standard of cleanliness and discipline at all times. Manure and effluent should be continuously disposal or treated in such a way as to minimize environmental contamination. Slurry should be stored for at least 3 months and bedding from the isolation areas is best burned. The current study was designed to understand the virulent genes in isolated *Salmonella* strains isolated from dairy farms [10-14].

### Materials and Methods

A total number of (95) samples were collected from three dairy farms at 3 different localities in Assiut Province including air (25), water (35) and manure (35) samples for detection of *Salmonella* species by routine bacteriological isolation and identification. Molecular characterization for detection of 6 virulence genes: *pefA*

(700 bp), *mgtC* (677 bp), *stn* (617 bp), *sopB* (517 bp), *invA* (284 bp) and *avrA* (422 bp) in *Salmonella* isolates.

### Air samples

Air Samples were collected using liquid impinges using sterile saline [15]. The samples were sent to the laboratory on ice without delay for further bacteriological examination. One ml from each sample was inoculated into Selenite F broth at 37°C for 24 hrs.

### Water samples

One liter from water was collected in a sterile glass bottles which sent to the laboratory on ice without delay [16]. In the laboratory, each water sample was filtered using membrane filter holder and filter papers (0.4 µm). The membrane filters were carefully transferred using a sterile forceps in to Selenite F broth and incubated overnight at 37°C for 24 hrs.

### Manure samples

250 g of manure was collected using sterile spatula in sterile plastic bags [1]. The samples were sent to the laboratory without delay. 90 g of each sample was suspended thoroughly in 900 ml sterile saline. The suspension was strained through narrow meshed sterile gauze into a sterile flask. 5 ml of the filtrate was inoculated into 20 ml of Selenite F broth and incubated at 37°C for 24 hrs.

### Bacteriological examination

On the next day, a loopful from the overnight inoculated Selenite F broth was streaked onto Brilliant green lactose phenol red agar, MacConkey's agar and *Salmonella-Shigella* agar and incubated at 37°C for 24-48 hrs. The suspected colonies were picked up and kept in slopes for further identification. Cultural and biochemical characteristics were used for identification of *Salmonella* [17-20].

### Serological examination

The cultures matching the biochemical reactions of *Salmonella* were further confirmed by the Kauffman-White scheme by the *Salmonella* Reference Laboratory at the Animal Health Research Institute, Dokki, Egypt [21]. According to Kauffman-White scheme, isolates were sero-

typed based on the agglutination tests on somatic "O" and phase 1 and phase 2 flagellar "H" antigens with antisera.

### Molecular characterization

Detection of *invA*, *sopB*, *mgtC*, *avrA*, *stn* and *pefA* genes in isolated *Salmonella* strains by PCR.

### Extraction of DNA

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

### Extraction of DNA

Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in Table (1).

### PCR amplification

Primers were utilized in a 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler [22,23].

### Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5 V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A gel pilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and 100 bp ladder (Fermentas) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Target gene	Primers sequences	Amplified segment (bp)	Primary	Amplification (35 cycles)			Final extension	References
			Denaturation	Secondary denaturation	Annealing	Extension		
invA	GTGAAATTATCGCCACGTTTCGGGCAA	284	94°C	94°C	55°C	72°C	72°C	Olivera et al.
	TCATCGCACCGTCAAGGAACC		5 min.	30 sec	30 sec	30 sec	10 min	
sopB	TCA GAA GRC GTC TAA CCA CTC	517	94°C	94°C	58°C	72°C	35	Huehn et al.
	TAC CGT CCT CAT GCA CAC TC							
avrA	CCT GTA TTG TTG AGC GTC TGG	422	94°C	94°C	58°C	72°C	35	Huehn et al.
	AGA AGA GCT TCG TTG AAT GTC C							
mgtC	TGA CTA TCA ATG CTC CAG TGA AT	677	94°C	94°C	58°C	72°C	35	Huehn et al.

	ATT TAC TGG CCG CTA TGC TGT TG								
Stn	TTG TGT CGC TAT CAC TGG CAA CC	617	94°C	94°C	59°C	72°C	35		
	ATT CGT AAC CCG CTC TCG TCC		5 min.	30 sec.	45 sec.	45 sec.			
pefA	TGT TTC CGG GCT TGT GCT	700	94°C	94°C	55°C	72°C	35		Murugkar et al.
	CAG GGC ATT TGC TGA TTC TTC C		5 min.	30 sec.	45 sec.	45 sec.			

**Table 1:** Primers sequences, target genes, amplicon sizes and cycling conditions.

## Results

Bacteriological examination of total examined 95 samples collected from 3 dairy farms in 3 different localities at Assiut Province revealed isolation of 3 (3.2%) *Salmonella* isolates from totally examined samples (table 1). The isolated organisms were identified serologically as 1 (2.9%) *Salmonella enterica* serotype Sandow isolated from water and 2 *Salmonella enterica* serotype Saintpaul, one from air (4.0%) and one from manure (2.9%). Beef cattle management practices.

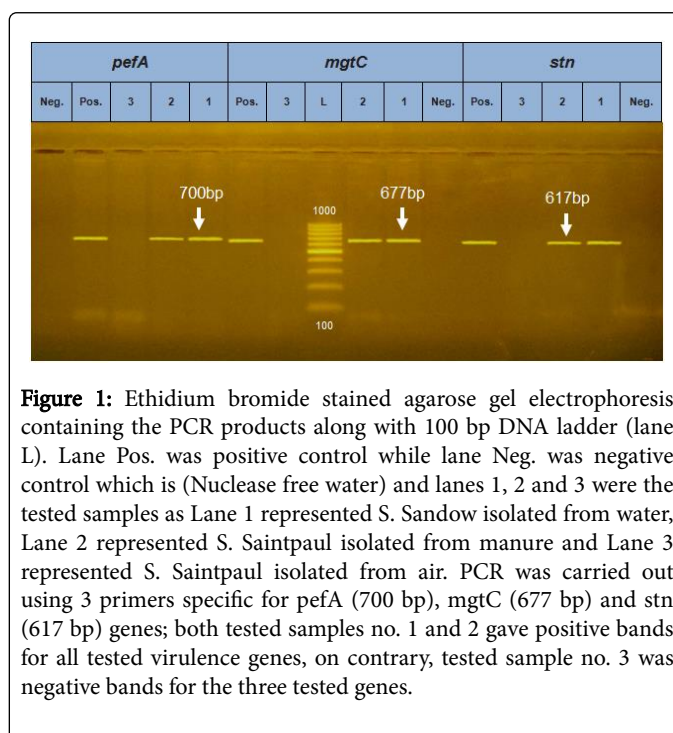
Samples	No. of examined samples	Bacteriological examination for <i>Salmonella</i>			
		Positive		Negative	
		No.	%	No.	%
Air	25	1	4	24	96
Water	35	1	2.9	34	97.1
Manure	35	1	2.9	34	97.1
Total	95	3	3.2	92	96.8

**Table 2:** Prevalence of *Salmonella* in different environmental samples.

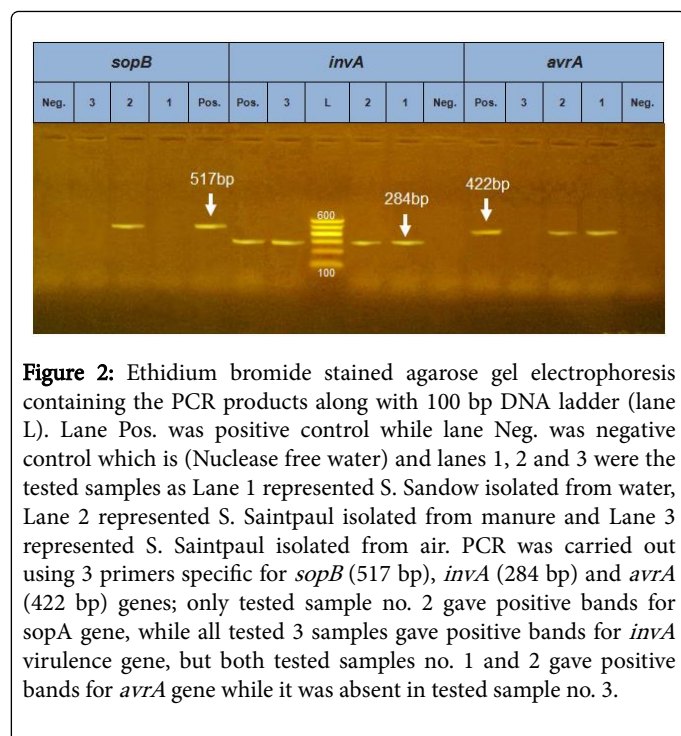
**Molecular characterization for the presence of 6 virulence genes; pefA (700 bp), mgtC (677 bp), stn (617 bp), sopB (517 bp), invA (284 bp) and avrA (422 bp) in *Salmonella* isolates revealed that all 6 tested virulence genes; except sopB (517 bp); presented in *Salmonella enterica* serotype Sandow isolated from water, while all 6 tested virulence genes found in *Salmonella enterica* serotype Saintpaul isolated from manure and found that invA (284 bp) was the only virulence gene located in *Salmonella enterica* serotype Saintpaul isolated from air.**

Sample No.	Source of sample	<i>Salmonella</i> isolate	Tested virulence genes					
			pefA	mgtC	Stn	sopB	invA	avrA
1	Water	S. Sandow	+	+	+	-	+	+
2	Manure	S. Saintpaul	+	+	+	+	+	+
3	Air	S. Saintpaul	-	-	-	-	+	-

**Table 3:** Molecular characterization of some virulence genes in *Salmonella* isolates.



**Figure 1:** Ethidium bromide stained agarose gel electrophoresis containing the PCR products along with 100 bp DNA ladder (lane L). Lane Pos. was positive control while lane Neg. was negative control which is (Nuclease free water) and lanes 1, 2 and 3 were the tested samples as Lane 1 represented S. Sandow isolated from water, Lane 2 represented S. Saintpaul isolated from manure and Lane 3 represented S. Saintpaul isolated from air. PCR was carried out using 3 primers specific for pefA (700 bp), mgtC (677 bp) and stn (617 bp) genes; both tested samples no. 1 and 2 gave positive bands for all tested virulence genes, on contrary, tested sample no. 3 was negative bands for the three tested genes.



**Figure 2:** Ethidium bromide stained agarose gel electrophoresis containing the PCR products along with 100 bp DNA ladder (lane L). Lane Pos. was positive control while lane Neg. was negative control which is (Nuclease free water) and lanes 1, 2 and 3 were the tested samples as Lane 1 represented *S. Sandow* isolated from water, Lane 2 represented *S. Saintpaul* isolated from manure and Lane 3 represented *S. Saintpaul* isolated from air. PCR was carried out using 3 primers specific for *sopB* (517 bp), *invA* (284 bp) and *avrA* (422 bp) genes; only tested sample no. 2 gave positive bands for *sopA* gene, while all tested 3 samples gave positive bands for *invA* virulence gene, but both tested samples no. 1 and 2 gave positive bands for *avrA* gene while it was absent in tested sample no. 3.

## Discussion

In many *Salmonella* outbreaks, infection was found to be widespread, with healthy animals excreting *Salmonella* in their faeces. Stressful events, such as parturition, nutritional stress, anorexia, other disease, etc., may trigger clinical disease. Since newly calved cows are susceptible, disease often occurs in calving boxes or stalls. These compartments should not be used for other animals until they have been thoroughly cleaned and disinfected [24]. Data illustrated in table (2) highlighted that bacteriological examination of total examined 95 samples collected from 3 dairy farms in 3 different localities at Assiut Province including air (25), water (35) and manure (35) samples revealed the isolation of 3 (3.2%) *Salmonella* isolates from totally examined samples. The isolates were identified serologically as 1 (2.9%) *Salmonella enterica* serotype Sandow isolated from water and 2 *Salmonella enterica* serotype Saintpaul, one (4.0%) from air and one (2.9%) from both manure. These results supported by findings of Abulreesh, 2012, who said that presence of *Salmonella* in water indicated fecal contamination.

Data found in Table 3, Figures 1 and 2 explored the molecular characterization for the presence of 6 virulence genes; *pefA* (700 bp), *mgtC* (677 bp), *stn* (617 bp), *sopB* (517 bp), *invA* (284 bp) and *avrA* (422 bp) in *Salmonella* isolates revealed that all 6 tested virulence genes; except *sopB* (517 bp); presented in *Salmonella enterica* serotype Sandow isolated from water. All 6 tested virulence genes were detected in *Salmonella enterica* serotype Saintpaul isolated from manure. Moreover, the *invA*(284 bp) was the only virulence gene located in *Salmonella enterica* serotype Saintpaul isolated from air. These results were similar to that published by who found that *Salmonella* serotypes differ in their natural reservoirs and ability to cause human infections [25-27].

## Conclusion

In addition to direct animal-to-animal transmission, environmental and management practices were identified as contributing to increase recycling of *Salmonella* in dairy farms. All fittings, utensils and surfaces should lend themselves to effective cleansing and disinfection. A strict hygiene routine for buckets and teats should be considered. Focused attention on determining sources of *Salmonella* infections will be vital to reduce *salmonella* infections.

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