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Prevalence, antibiogram, molecular characterization and reduction trial of *Salmonella typhimurium* isolated from different fish species

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Abstract

***Salmonella* is a major cause of food-borne outbreaks and infections in many countries worldwide. The present work aimed to investigate the prevalence, antibiotic sensitivity and detection of virulence-associated genes in *Salmonella typhimurium* isolated from fish samples (tilapia, mullet and catfish) collected from Zagazig city markets, Egypt. *Salmonella typhimurium* was isolated only from tilapia with a percentage of 13.3%. Antibiotic susceptibility testing of *Salmonella typhimurium* revealed marked susceptibility to ofloxacin. Two different virulence genes (*ssa*P and *pip*B) were expressed in the isolated *Salmonella typhimurium*. Dipping of tilapia in liquid smoke 5% for 30 min reduced *Salmonella typhimurium* count by 40%. In conclusion, our results confirm contamination of tilapia by *Salmonella typhimurium*. Dipping of fish in liquid smoke is an efficient strategy in reducing *Salmonella typhimurium* load in fish.**

Keywords: Antibiotic resistance, fish, *Salmonella*, virulent genes, liquid smoke.

Introduction

The demand of fish consumption has increased continuously for many communities because of its low price, low cholesterol level and high protein and energy sources when other sources of animal protein are in a short supply. Fish is one of the most highly perishable food products but during handling and storage, quality deterioration of fresh fish rapidly occurs and limits the shelf life of the product[9)].

Salmonellosis is a worldwide disease; it is estimated to cause more than 1.2 million illnesses each year in the United States alone, with more than 23,000 hospitalizations and 450 deaths[5)]. Infection of humans by *Salmonella typhimurium (S. typhimurium)* generally results in gastroenteritis, severe abdominal cramping, fever, weakness and severe diarrhea[13)]. Attention is being paid for detection of *Salmonella* pathogenicity islands (SPI-2 and SPI-5) coding virulence genes (*ssaP* and *pipB*) associated with *S. typhimurium*[4)]. Liquid smoke has traditionally been used as a flavoring agent in protein-based foods, known to possess antioxidant properties, and serves as a natural alternative to conventional

antimicrobials[14].

Thus, the present study was designed to investigate the prevalence of *Salmonella* species in the most commonly consumed fish (tilapia, mullet and catfish) in Zagazig city, Egypt. Furthermore, antibiogram and virulence-associated genes of *S. typhimurium* were also studied. Moreover, a reduction trial of *S. typhimurium* load and extension of shelf life of tilapia fish were conducted using liquid smoke in a comparison with potassium sorbate.

Materials and methods

Chemicals & reagents:

Peptone and potassium sorbate were from Merck, Germany. Rappaport Vassiliadis (RV) broth and xylose lysine desoxycholate (XLD) media were purchased from Oxoid, CM469, Adelaide, Australia. The ready-made liquid smoke was kindly gifted from Food Science and Technology Research Institute, Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt.

Sampling & Sample preparation:

Ninety samples of tilapia (*Oreochromis niloticus*), mullet (*Mugil cephalus*) and catfish (*Clarias lazera*) (30 of each) were randomly collected from different fish shops at Zagazig city, Egypt. Samples were transferred rapidly to the laboratory under aseptic condition for bacteriological examination.

Twenty-five grams from each sample were aseptically transferred into a sterile blender containing 225 ml of sterile peptone water 1%. The homogenate was incubated at 37 °C for 24h for pre-enrichment of the samples.

Isolation & identification of Salmonella species:

Isolation and identification of Salmonella species was carried out according to the method described before[15]. Briefly, one ml of pre-enriched peptone water was enriched in RV broth and then streaked onto XLD agar plates. Plates were incubated at 37 °C/48 h and red colonies with black center were enumerated (plating was performed in triplicate)

The obtained purified isolates were identified biochemically using Oxidase test, hydrolysis of urea, H2S production and Utilization of citrate. The biochemically identified Salmonella isolates were subjected to serotyping following Kauffman White scheme with commercial antisera (Difco Laboratories Deteroeit, Mitchigeu, USA) for cell wall (O) and Flagellar (H) antigen identification.

Antibiotic susceptibility test:

S. typhimurium isolates were tested for their susceptibility to six antibiotics by disc diffusion method on Muller-Hinton according to the recommendations of National Committee for the Clinical and Laboratory Standards. The antibiotics used were Ciprofloxacin (10 μg), Gentamicin(10 μg), Clindamycin (2 μg), Penicillin (5 μg), Chloramphenicol (30 μg), Doxymcin(10 μg) and Ofloxacin(5 μg). These antibiotics are frequently used to treat Salmonella Typhimurium food poisoning in Egypt.

DNA preparation:

Bacterial DNA was extracted from each of glycerol stock serodiagnosed *S. typhimurium* according to the method described before[3]. DNA concentration in supernatant was evaluated by Nanodrop (ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

Detection of virulence factors by Polymerase Chain Reaction (PCR):

Tested *S. typhimurium* were examined for harboring ssaP and pipB of two pathogenecity islands for virulent determination of *S. typhimurium* using the primer sequences and the method published before[3].

Extension of shelf life and reduction of S. typhimurium count using liquid smoke and potassium sorbate:

Purified *S. typhimurium* strain was adjusted to106 cfu/ml according to the previous method[10] and used for the reduction trial experiment.

Five hundred grams of fresh tilapia fish muscle were divided into five equal groups; each group consists of five samples (20 g each).

Artificial contamination of fish samples with S.

typhimurium:

Fish samples were dipped separately in 100 ml sterile peptone water 0.1% containing *S. typhimurium* (10^6 cfu/ml). The inoculated fish samples were left for 30 min at room temperature (25 °C) to allow attachment and absorption of the inoculated bacteria. The contaminated samples were divided into the following groups:

First control group: non-treated; 2nd group: treated with potassium sorbate 1%; 3rd group: treated with potassium sorbate 5%; 4th group: treated with liquid smoke 3% and 5th group: treated with liquid smoke 5%.

All groups were packed in a separate sterile polyethylene bags and stored in refrigerator at 4±1 °C. Sensory analysis (color, odor and texture) was conducted every 24 h during storage[12]. *S. typhimurium* count was conducted every 48 hrs during storage.

Statistical analysis: Statistical significance was evaluated using Tukey-Kramer HSD test using (JMP statistical package, SAS Institute Inc., Cary, NC, USA). A P-value<0.05 was considered significant.

Results and Discussion

In this study, the overall percentage of Salmonella species isolation was 20% (Table 1). *Salmonella serovar typhimurium* was the clinically important serovar mostly identified in this study, which indicates its ability to adapt and survive in the environment[2]. *S. typhimurium* was only isolated from tilapia with a percentage of 13.3% (Table 1). This result goes in line with David *et al*.[7] who could isolate *S. typhimurium* from tilapia species harvested from Lake Victoria, Kenya with a percentage of 11.1%. Regarding other *Salmonella* serovars, *S. rubislam* was isolated from 6.7% of tilapia samples. In mullet samples, *S. rubislam* and *S. senegal* were isolated with percentages of 20% and 6.7% respectively. In catfish samples, *S. senegal* was isolated with a percentage of 13.3% (Table 1). *Salmonella* may be the foodborne pathogen most likely associated with catfish [11]

Table 1: Prevalence of Salmonella species in the examined fish species (n=30)

Fish samples	*Salmonella* species			
	S. typhimurium	*S. rubislam*	*S.senegal*	*Overall*
Tilapia	4 (13.3%)	2 (6.7%)	-	6 (20%)
Mullet	-	6 (20%)	2 (6.7%)	8 (26.66%)
Catfish	-	-	4 (13.3%)	4 (13.3%)
Total (90)	4 (4.4%)	8 (8.9%)	6 (6.66%)	18 (20%)

The widespread use of antibiotics in growth promotion and disease control has resulted in drug-resistant of *Salmonella* strains *typhimurium* to many antibiotics such as penicillin, low sensitivity to clindamycin and doxymycin, moderate sensitivity to gentamycin and ciprofloxacin and high sensitivity to chloramphenicol and ofloxacin (Table 2).

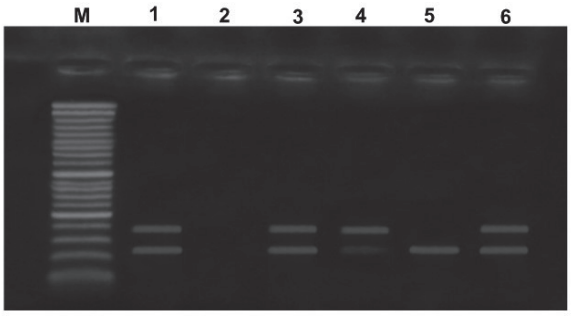
Table 2: Antibiogram of the isolated *S. typhimurium*

Antibiotic	Susceptibility of *S. typhimurium*	%
Ciprofloxacin	Moderate sensitivity	100
Gentamycin	Moderate sensitivity	100
Clindamycin	low sensitivity (+)	100
Penicillin	Resistant	100
Chloramphinicole	High sensitivity (+++)	100
Doxymycine	low sensitivity (+)	100
Ofloxacin	High sensitivity (+++)	100

The results achieved in this study showed a clear resistance of *S. typhimurium* to penicillin, low sensitivity to clindamycin and doxymycin, moderate sensitivity to gentamycin and ciprofloxacin and high sensitivity to chloramphenicol and ofloxacin (Table 2). These findings are in agreement with Darwish *et al.* [6] who reported a marked susceptibility of *S. typhimurium* to fluoroquinolones (ofloxacin andciprofloxacin). Thus, these antibiotics are recommended to be used in the treatment of infections caused by this organism.

Salmonella has a large number of genes, which implicated in salmonella virulence. Several of these virulence determinants are clustered within pathogenicity islands. The expression of two different virulence associated genes (saaP and pipB) which coded by two pathogenicity islands (SPI-2 and SPI-5) were examined in the identified *S. typhimurium* isolates. In the present study, *pip*B gene was detected in all of the isolated *S. typhimurium,* but *ssaP* was expressed in three out of the four isolates (Fig. 1). These results were in line with Fazl *et al.*[8] who detected both *pipB* and *ssaP* genes in 100% of the obtained *Salmonella* isolates in Iran. *pip*B gene is targeted to detergent–resistant microdomains of intracellular membranes, which leads to the speculation of a possible interaction between *pip*B and host cell signaling molecules4). However, *ssa*P is dedicated for virulence, which confirms the importance of these genes at different stages of infection and for survival of bacteria inside the host cells[3].

Figure 1: Expression of virulence associated genes in S. typhimurium isolates



A multiplex PCR for the expression of *pipB* (230 bp) and *ssaP* (375 bp) virulence genes in *S. typhimurium* isolates. M: 100 bp ladder, lanes 1: positive control, 2: negative control, 3-6: *S. typhimurium* isolates

As a trial for extension of the shelf life of the fish and reducing the load of *S. typhimurium*, liquid smoke was compared with potassium sorbate for their protective effects. Dipping of tilapia fish in liquid smoke 5% for 30 min was the most effective to extend the shelf life until 17 days storage at 4 °C compared with the control and to reduce S. typhimurium by 2.6-log cfu/g (40 %), followed by potassium sorbate 5% and liquid smoke 3% (Table 3 & 4). In agreement with our results, Achmadi *et al.*[1] reported that liquid smoke is able to extend the shelf life of fish ball and fresh fish through reducing moisture content and via bacteriostatic effects of the phenolic compounds naturally found in the smoke.

In addition, liquid smoke probably acts on the microbial cell membrane affecting the stability of the bacteria. The antimicrobial activity of liquid smoke may be attributed to the presence of antimicrobial and antioxidant compounds like aldehydes, carbonyls, carboxylic acids, organic acids and phenols[14]. In conclusion, the results obtained in this study declared that fish might be a possible reservoir for S. typhimurium. Thus, efficient cooking of fish and fish products and hygienic handling, trans-port, commercialization and storage should be carried out.

Table 3: Effects of liquid smoke and potassium sorbate treatment on sensory parameters of tilapia during cold storage at 4 °C

Storage period	1st day	3rd day	5th day	7th day	9th day	11th day	13th day	15th day	17th day	15th day
Control	6	4	3	Spoiled	-	-	-	-	-	-
S 1%	8	7	6	4	3	Spoiled	-	-	-	-
S 5%	9	8	8	7	7	6	6	5	4	spoiled
LS 3%	8	7	7	6	6	5	5	4	Spoiled	-
LS 5%	9	8	8	7	7	6	6	5	4	spoiled

Table 4: Effects of liquid smoke and potassium sorbate treatment on *S. typhimurium* count (Mean ± SD log cfu/g) during cold storage at 4 °C

Groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day	15th day	17th day	19th day
Control	6.5±4.9	6.9±4.9	7.2 ±6.3	Spoiled	-	-	-	-	-	-
S 1%	6.3± 5.1	5.7±3.9	4.6±3.2*	4.6±3.2	4.3±3	Spoiled	-	-	-	-
S 5%	6.1± 5.1	5.5±3.9	4.6±3.3*	4.6±3.1	4±3.1	4.01±2.9	4.4±2.9	4.6±2.8	5.7±4.1	Spoiled
LS 3%	5.8±3.9	4.2±3.1*	4.0 ±3.2*	4.02 ±3.1	4.3±2.9	4.3±2.9	4.4±2.9	4.02±2.9	Spoiled	-
LS 5%	5.6±3.9	4.0 ±2.9*	4.0 ±3.2*	4.01 ±3.2	4±2.9	4.01±2.9	4.01±2.9	3.7±2.1	3.9±2.1	Spoiled

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