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Evaluation of the Hygienic Status of Some Fish Types

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ABSTRACT

This study was conducted to evaluate the level of microbial contamination in three types of chilled fish that are sold in Zagazig markets [grey mullet (*Mugil cephalus*), Tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*)]. A total of 150 fish samples belonging to the 3 species (50 of each) were examined for *S. aureus*, Coliforms, and *E. coli*. The incidence of *S. aureus* in examined samples were (20%, 28% and 42%) respectively, while the range of *S. aureus* count in the examined fish samples ranged from 2.39 to 3.52, 3.58 to 4.23, and 3.59 to 4.91log¹⁰ CFU/g respectively. The coliforms count in examined fish samples ranged from 2.04 to 3.66, 1.47 to 4.04 and 1.56 to 4.04log¹⁰ CFU/g respectively. The incidence of *E. coli* in examined samples were (8%, 12% and 22 %) respectively. Staphylococcal enterotoxin gene (*coa*) and virulence genes (*stx1* and *stx2* genes) in *E. coli* isolates were determined by multiplex PCR. Three isolates of *E. coli* found to harbor *stx2* gene while *stx1* could not be detected in all examined isolates. *Coa* gene was detected in five examined isolates of *S. aureus*. The present findings concluded that those fish species could constitute a public health hazard. This study highlighted the importance of screening of fish for enterotoxigenic *S. aureus* strains and STEC isolates, and also assessing the hygienic quality of fish.

INTRODUCTION

Fish has high consumer preference due to its inherent nutritive value, taste and easy digestibility. It is one of the most important sources of animal protein available and has widely been accepted as a good source of pro-

tein and other elements for maintenance of healthy body (Andrew, 2001).

One of the essential things in food hygiene is the examination of food, especially for the presence of microorganisms. Fish are often infected with or may harbor various bacteria

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which may cause health hazards to the fisherman, fish handlers and even to the consumers (Razavilar et al .2013).

A heterogeneous group of microorganisms are usually reported to be associated with different body parts of fish. Among the many pathogens associated with fish foods, *E. coli* have been identified as important one and is present as normal flora in lower intestine of man as well as animals. *E. coli* is considered major microbial pollutant of water and originates due to contamination of various water with human and animal excreta. Fish and fish products usually acquire contamination from surrounding polluted water or from usages of non-potable water in their preparation and processing. Wide variety of infections are caused by *E. coli* in human including foodborne illnesses that may range from diarrheal disease to life threatening hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) due to shiga toxic *E. coli* (James et al. 2001).

E. coli is not a natural inhabitant of the fish microbiota, nevertheless, it can be isolated from their gut due to its presence in contaminated aquatic environments (Guzman et al. 2004). It is worth noticing that this microorganism have pathogenic strains standing out as emerging zoonotic potential, as well as shiga toxigenic (STEC) and enteropathogenic (EPEC) *E. coli*. STEC strains produce the shiga toxin (*Stx*), which is representing the main virulence factor. There are two classes of shiga toxin, *Stx1* and *Stx2*, with the last one presenting seven subtypes (Scheutz et al. 2012)

Some bacteria such as Staphylococcal spp. may be found only in the body surface (Ekpo et al. 2010). The microbial count in different fishes have showed that the number of organisms were more on the body surface of big fishes with higher body weight rather than on the surface of small fishes, because the organisms use the surface area of the fish as a microhabitat in their ecosystem. After fish is being caught and dying, the immune system collapses and bacteria are allowed to proliferate freely on the skin surface (Emikpe et al. 2011).

The present study was undertaken to deter-

mine the incidence of *S. aureus*, coliform and *E. coli* to evaluate the hygienic quality of 3 different fishes marketed in Zagazig local fish market.

MATERIALS And METHODS

1. Samples Collection: A total of 150 fresh fish samples [Grey mullet (*Mugil cephalus*), Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*)] 50 for each were purchased from different fish markets with variable degree of hygienic conditions present in Zagazig City. All collected samples were immediately transferred in ice-box container, aseptically handled and moved promptly to microbiology laboratory at Animal Health Research Institute, Zagazig Lab., to be evaluated bacteriologically.

2. Preparation of samples (APHA 2001): Ten grams of each sample were taken under aseptic conditions, transferred into a sterile polyethylene bag to which 90 ml of 0.1% Sterile buffered peptone water (Oxoid CM9) was aseptically added to provide a dilution 1/10, then the content was homogenized for not more than 2.5 minutes using blender at high speed not less than (2000 r.p.m). Then the mixture was allowed to stand for 15 minutes at room temperature. The contents of the jar were mixed by shaking before applying the following technique.

One ml from the original suspension (10^{-1}) was transferred aseptically into sterile test tube containing 9 ml of sterile peptone water 0.1 % to obtain a dilution of (10^{-2}) from which further 10 fold decimal dilution were prepared up to suitable countable dilution.

3. Determination of *S. aureus* counts: according to FDA (2001)

One ml of the prepared dilution was poured into plates containing Baird Parker media with Egg yolk-Tellurite emulsion incubated at 35 °C, and observed after 48 hours. Characteristic black colonies surrounded by a narrow white margin with a zone of clearing were counted to obtain the total *S. aureus* counts per gm.

Confirmation of *S. aureus* was carried out by using the following biochemical tests: Gram

staining, Catalase test, Coagulase test and detection of hemolysis.

4-Total coliform enumeration: those counts were carried out according to (FDA, 2002) using 3 consecutive dilutions, 1 ml aliquots from each dilution was inoculated into 3 Lauryl sulfate tryptose broth (LST) tubes containing Durham's tubes for a 3 tube MPN analysis. Lauryl tryptose broth (LST) tubes were incubated at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 48 h. Positive tubes showing gas production in the Durham's tubes were recorded and the MPN for coliforms were calculated.

a-MPN - Confirmed test for coliforms:

From each gassing LST broth tube, a loopful of suspension was transferred to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and examine for gas production at 48 ± 3 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

4. Escherichia coli (Priyanka and Alka 2008) After samples were inoculated into buffered peptone water, the homogenate were incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 hrs. By using sterile loop the pre-enrichment broth culture cultivated into eosine in methylene blue agar plates. The inoculated plates were incubated at 37°C for 24 hrs.

6- Detection of Toxin producing genes in isolated *S. aureus* and *E.coli* strains using PCR:

Five of each *S. aureus* and *E.coli* isolates with a sequence of 1,2,2 samples from Grey mullet, Tilapia, African catfish respectively were subjected to DNA extraction. 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 pro-

vided in the kit.

Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in the next table.

PCR amplification.

***Staph .aureus* Coa gene:** Primers were utilized in a 25- μl reaction containing 12.5 μl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 μl of each primer of 20 pmol concentration, 4.5 μl of water, and 6 μl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

***E. colistx1* and *stx2*:** Primers were utilized in a 50- μl reaction containing 25 μl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 μl of each primer of 20 pmol concentration, 13 μl of water, and 8 μl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

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 5V/cm. For gel analysis, 20 μl of each PCR product were loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo Scientific, Germany) was 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.

Table (A). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>coa</i>	ATA GAG ATG CTG GTA CAG G	630	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	Iyer and Kumosani, 2011
<i>Stx1</i>	GCT TCC GAT TGT TCG ATG C ACACTG- GATGATCTCAGTGG CTGAATCCCCCTCC ATTATG	614	94°C 5 min.	94°C 30 sec.	58°C 45 sec.	72°C 45 sec.	72°C 10 min.	Dipineto et al., 2006
<i>Stx2</i>	CCATGACAAC- GGACAGCAGTT CCTGTCAACTGAG- CAGCACTTTG	779						

RESULTS

Table (1) Incidence and count of *S. aureus* (Log₁₀ CFU/g) in examined fish samples. (n= 50 for each fish type)

Fish sampled	Positive samples		<i>S. aureus</i> count (cfu/g)		
	No	%	Min.	Max.	Mean ±S.E.
Grey mullet	10	20	2.39	3.52	2.92 ±0.14
Nile tilapia	14	28	3.58	4.23	3.84 ± 0.06
African catfish	21	42	3.59	4.91	4.23± 0.11

S. aureus count (log₁₀ CFU/g) of positive examined fish samples.

Table (2) Incidence and count of Coliforms (log₁₀CFU/g) in examined fish samples.

Fish sampled N= (50 for each)	Positives Samples		Coliforms count (Log ₁₀ cfu/g)		
	No	%	Min.	Max.	Mean ±S.E.
Grey mullet	13	26	2.04	3.66	3.30 ± 0.21
Nile tilapia	18	36	1.47	4.04	1.83 ± 0.22
African catfish	20	40	1.56	4.04	± 0.182.85

Coliforms count (log₁₀CFU/g) of positive examined fish samples.

Table (3). Frequency distribution of *S. aureus* and Coliforms count with maximal permissible limits in the examined sample types (n= 50 for each fish type).

Micro-organisms	P.L.* CFU/gm	Grey mullet						Nile tilapia						African catfishs					
		Not Detected		Within P.L.*		Over P.L.		Not Detected		Within P.L.		Over P.L.		Not Detected		Within P.L.		Over P.L.	
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
<i>S. aureus</i>	10 ⁵	40	80	6	12	4	8	36	72	0	0	14	28	29	58	0	0	21	42
Coli-forms	10 ²	37	74	1	2	12	24	32	64	3	6	15	30	30	60	0	0	20	40

*: ES (2005) Chilled fish /3494

S. aureus P.L according to ES (2005) : 3 log₁₀ CFU/g

Coliform P.L according to ES (2005) : 2 log₁₀ CFU/g

Table (4) Incidence of *E. coli* in examined fish samples (n= 50 for each fish type).

Fish sampled	No. of positives	Percentage Incidence%
Grey mullet	4	8
Nile tilapia	6	12
African catfishs	11	22

Table (5) Occurrence of Coa gene of *S. aureus* isolated from examined fish samples

<i>S. aureus</i> sample	Coa
1	+
2	+
3	+
4	+
5	+

100% of examined *S.aureus* strains isolated from fish samples harbor Coa gene

Photo(1). Electrophoretic pattern of coagulase (*coa*) gene in different *S.aureus* isolates.

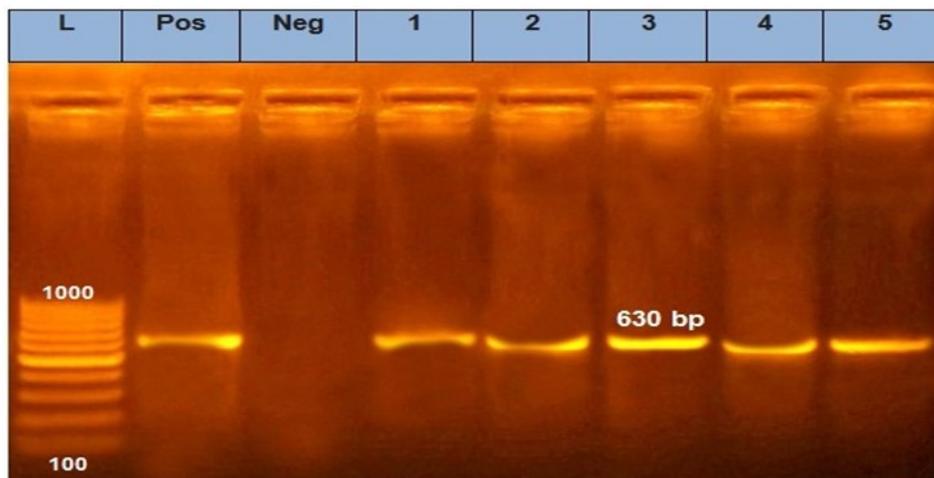
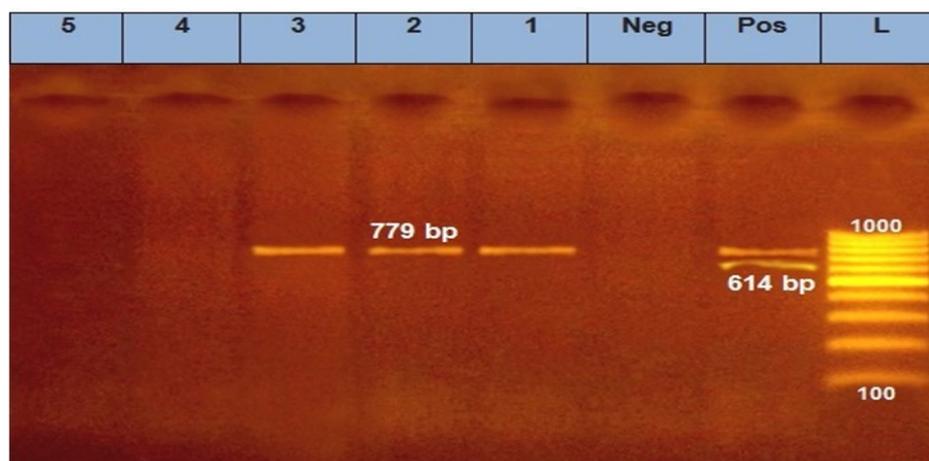


Table (6) Occurrence of *Stx1* and *Stx2* genes of *E. coli* isolated from examined fish samples

<i>E. coli</i> sample	Results	
	<i>Stx1</i>	<i>Stx2</i>
1	-	+
2	-	+
3	-	+
4	-	-
5	-	-

Stx1 not detected in all examined *E. coli* isolates, while 60% harbor *Stx2*

Photo (2) Electrophoretic pattern of *stx1* and *stx2* genes in different *E. coli* isolates

DISCUSSION

The obtained data in Table 1 exhibited that, among the different varieties of fish, African cat fish showed the highest *S. aureus* contamination (42%) followed by Tilapia (28%) and Grey mullet (20%). The results correlates with earlier works by Ali (2014); while, El-Olemy et al. (2014) recorded lower results as they could isolate *S. aureus* in percent of 3 and 6 % from Tilapia nilotica and African catfish, respectively. The *S. aureus* count in the examined fish samples ranged from 2.39 to 3.52, 3.58 to 4.23, and 3.59 to 4.91 log¹⁰ CFU/g respectively.

During transportation, periodical dampening of fish with contaminated water is used to prevent over heat and drying. It was observed that the cumulative effect of such conventional practices coupled with unhygienic handling during transportation could result in high level of *S. aureus* in marketed food fish. Lack of proper drainage facilities and heavy fly infestation in the Indian market also promotes tertiary contamination to a great extent (Bujamma and

Padmavathi 2015)

Concerning coliforms count listed in table (2) they are ranged from 2.04 to 3.66, 1.47 to 4.04 and 1.56 to 4.04 log₁₀ CFU/g, in grey mullet, Tilapia and African catfish, respectively.

Presence of coliform bacteria in the samples indicates poor hygiene and sanitary conditions of the fish. Yagoub (2009) isolated coli forms from fresh water fish muscle with count ranged from 2–4.6 log₁₀ CFU/g and Budiati et al. (2015) found that the coliform count were ranged from 1.46 to 4.18 log MPN gr⁻¹ for Catfish, 1.6 to 4.04 log MPN gr⁻¹ for tilapia. These results are nearly in correspondence with the present findings.

The contamination of fishes with coliforms may be due to environmental conditions during transportation to landing centers and whole sale markets through handling process that may be associated with infection (Das et al. 2007, Begum et al. 2010).

The obtained data in Table (3) exhibited that 92%, 72%, 58% of the examined Grey mullet, Nile tilapia and African catfish respectively showed that *S. aureus* counts were for both negative and with in the P.L. samples according to E.S (2005) , while 8%,28%,42% were unaccepted based on their *S. aureus* count according to E.S No. 3494 (2005).

Eltholth et al. (2018) mentioned that only 13% of the collected tilapia fish samples showed higher MPL stated by E.S No. 3494 (2005) for *S. aureus*.

In the present study 76%,70% and 60% of the examined Grey mullet, Nile tilapia and African catfish respectively showed that coliforms counts were within the P.L. according to E. S No.3494 (2005) and 24%, 30%, 40% of the examined samples showed that coliforms counts were exceeded the P.L. according to E. S No. 3494 (2005) .

The permissible limit of total coliform count is 10^2 MPN/gm as recommended by ES No.3494 (2005).

The incidences of *E. coli* isolated from Mugil cephalus, Tilapia and African catfish samples as recorded in table (4) were 8, 12 and 22%, respectively. *E. coli* could be isolated from fresh water fish muscle in percentage of 48.95% by **Gupta et al. (2013)**. This finding is higher than the present study while nearly similar results were reported by **Yagoub, (2009)** who reported that 13% of examined fresh water fish were contaminated with *E. coli* and also **Lobna and El- Newishy (2010)** could isolate the organism from Tilapia and African catfish samples with incidence of 14.3 and 22.9%. Lower result was reported by **Rocha et al. (2014)** who found that 4 (9.09%) of the isolates were confirmed to be *E. coli* from fresh water fish muscles.

E. coli is a common disease of freshwater fish especially under culture conditions and play a big role in economic losses to fish industry (**Baya and White 1997**). Bacterial diseases in fish generally do not develop simply as the result of exposing a host to an infectious agent. In most instances, disease occurs as the result of complex interactions between pathogen, fish and environmental stress, which affect the susceptibility of the host to disease

(**Wedekind et al. 2010**).

In this study *coa* gene (Table 5) was detected in all five examined isolates of *S. aureus* and give a single amplicon of 630 bp as shown in fig. photo (1) .

Coagulase production is an important phenotypic determinant of *S. aureus* which it is associated with virulence as it resists phagocytosis and strong than the bacterial virulence (**Bhanderi et al. 2009**).

To assess the virulence potential of the *E. coli* virulence genes (Table 6), isolates PCR protocols were optimized targeting two well-known virulence genes-*stx1* and *stx2* as described previously. *Stx1* could not be detected in any of examined *E. coli* isolates while *Stx2* detected in (60%) 3 out of 5 isolates.

Amplification of *stx2* genes resulted in a single amplicon with a size of approximately 779 bp, for *E. coli* strains indicating this gene as in photo (2).

Higher results were tabulated by **Gupta et al. (2013)** who detected *Stx1* and *Stx2* in percent of 72.2 and 51.8 % of examined *E. coli* isolates.

Conclusion

In the present study *S. aureus* and *E coli* were detected in some of the collected samples of raw fish sold at domestic fish market in Sharkia governorate. Fish marketing systems should be maintained clean with improvements in handling and processing to minimize the prevalence of pathogenic bacteria. In order to provide quality fish to the consumers, strict hygienic practices should be followed in fish markets. The bacteriological qualities of these fishes could be substantially worse after unhygienic fish handling ,cleaning , purchase in the local retail markets and subsequently this may constitute a health hazard for consumers. Thus, it is recommended that a thorough surveillance of the microbiological status of fish to be done for the safety of the ultimate consumers.

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