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Occurrence and characterization of *Pseudomonas* species isolated from Fish Marketed in Sohag Governorate, Egypt

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Abstract

The aims of this study were isolation and characterization of *Pseudomonas* spp. from fish meat collected from Sohag governorate, Egypt. A total 120 fish samples including frozen mackerel, frozen saurus, chilled *Mugil cephalus* and chilled *Tilapia nilotica* (30 of each) were collected from different shops and supermarkets in Sohag governorate. *Pseudomonas* spp. were isolated from 65% of the examined samples. The obtained data revealed that the highest count of *Pseudomonas* was in chilled Tilapia nilotica. The prevalence of *Pseudomonas aeruginosa* in frozen mackerel, frozen saurus, chilled Tilapia nilotica and chilled Mugil cephalus was 33.3%, 30%, 23.3% and 26.6% respectively. Furthermore, psychrotrophic count was performed and the results demonstrated that it was the highest in frozen mackerel followed by *Tilapia nilotica* and the *Mugil cephalus* showed the lowest count. Furthermore, the occurrence of *oprL*, *phzM* and *toxA* virulence genes was studied in some selected isolates by PCR. The findings showed that all the selected isolates possessed the virulence genes. This work showed contamination of fish samples with *Pseudomonas* spp., indicating the importance of applying hygienic measures during handling and storage of fish.

Keywords:

Pseudomonas aeruginosa, Contamination, PCR, Virulence genes.

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Introduction

Fish and seafood represent an essential and famous food for several parts of the world population and in some countries, fish is considered the main source of animal protein (Allison et al., 2009). Moreover, fish is known as an inexpensive source of protein compared with other protein products like beef and poultry

Various bacterial diseases can infect a large variety of fish and initiate significant financial damage. The loss is related to bad growth, deaths and inferior meat quality. *Pseudomonas* is one of the most common fish bacterial diseases. *Pseudomonas* is a part of the usual fish microflora and can be opportunistic and developed into virulent and disseminated in distressed fish. *Pseudomonas* plays a role in the process of fish decomposition and in some circumstances, they may become human pathogens and induce infection particularly human infection caused by *Pseudomonas aeruginosa* (Zilberberg and Shorr, 2009). *Pseudomonas aeruginosa* can cause serious diseases in exhausted fish including, hemorrhagic septicemia, congested kidney, gill necrosis and friable liver (Ardura et al., 2013).

The occurrence of *Pseudomonas* in fish has been reported in many countries (Yagoub 2009; Ardura et al., 2013; Algammal et al., 2020). Currently, extreme efforts for diagnosis of *Pseudomonas aeruginosa* have been recognized, not only because of its economic value, but because of its public heath significance. *Pseudomonas* spp. is currently listed as a foodborne illness that affects consumers through consumption and handling infected fish (Gram et al., 2002).

Sohag is placed on the western side of the Nile, on a productive soil region. People in Sohag are accustomed to purchasing raw fish from markets and street vendors rather than purchasing cooked fish. The purpose of the present work was to study the existence of *Pseudomonas* spp. in sample of commercial fish offered in Sohag market, Egypt. In addition to, genotypic analysis of certain virulence genes was conducted to examine their probability of bacterial pathogenicity.

Materials and methods

Fish sampling:

A total of 120 random samples of local frozen Mackerel, frozen Saurus chilled *Mugil Cephalus* and chilled *Tilapia nilotica* (30 of each) were randomly collected from various fish markets located at Sohag governorate, Egypt. The samples were placed in an ice box until performing bacteriological examination in the laboratory.

Enumeration and identification of Pseudomonas:

Twenty-five g from flesh of fish was homogenised with 225 ml of peptone water, serial decimal dilutions were carried out. 100 µL of each dilution was spreads *Pseudomonas* agar base media and incubated at 25ºc for 48 h and the devolved colonise were counted (Roberts and Greenwood, 2003) Furthermore, colonises were collected and purified and subjected for identification by Gram stain and conducting biochemical tests as described by Quinn et al. (2002), Austin and Austin (2007).

Enumeration and identification of psychrotrophic bacteria:

One-hundred µL of each dilution was spread onto plate count agar and incubated at 7ºc for 10 days. Furthermore, isolation and identification of Gram-positive and Gram-negative bacteria was performed as described by Quinn et al. (2002), Barrow and Feltham, (2004). Briefly, samples were inoculated into nutrient broth at 37ºfor 24 hours, then a loopful was streaked into blood agar for isolation of Gram-positive

bacteria, Baird Parker Agar for isolation of *Staphylococci* spp., tryptone Soya agar for isolation of *Bacillus* spp., MacConkey agar for isolation of Gram-negative bacteria. Identification of bacteria was performed according to their colony features, Gram's staining and various biochemical reactions.

Molecular typing of the virulence genes of the isolated Pseudomonas aeruginosa:

Some strains of *Pseudomonas aeruginosa* were selected for detection *oprL*, *toxA* and *phzM*. DNA was extracted by boiling method as described by Reischl et al. (2002). The three sets of primers (Table 1) were used for detection of virulence genes. Each PCR reaction was done in a total volume of 20µl as follows: 2 µl of template DNA, 0.6 µl MgCl2, 0.4 µl of each primer, 0.2 µl dNTP, 2µl of 10 x PCR buffer, 0.5 µl of Taq DNA polymerase (5U/µl) (SinaClon, Tehran, Iran) and 13.9 µl of Milli-Q water. The PCR condition was an initial denaturation at 95ºc for 5 minutes, then processed into 35 cycles each one was denaturation at 95ºc for 30 second, annealing at 55 ºc for 35 seconds and extension at 72 ºc for 30 second. A PCR reaction without any DNA was utilized as a negative control, while a reference strain of *Pseudomonas aeruginosa* gladly given by the Animal Health Institute in Giza, Egypt, was utilized as positive control.

Table 1. Primers used for the amplification of different virulence genes among *Pseudomonas aeruginosa* isolates.

Target gene	Primer sequence	Size	Reference
oprL	F: CGGGCGTGCTGATGCTCGTAT R: GCGCGAG GAACGTCAGGACAC	709 bp	Vijayakumar et al., 2011
toxA	F:GACAACGCCCTCAGCATCACCAGC R:CGCTGGCCCATTCGCTCCAGCGCT	396 pb	Verove et al., 2012
phzM	F: CCGTCGAGAAGTACATGAAT R: CATAGTTCACCCCTTCCAG	857 pb	Sambrook et al., 1989

Results

Bacteriological assay:

The bacteriological examination for the occurrence of *Pseudomonas* revealed that *Pseudomonas* spp. were isolated from 65% of examined samples. *Mugil cephalus* showed a high contamination level (73.3%) followed by *Tilapia nilotica* (66.6%) while saurus demonstrated a low contamination level (56.6%). Notably, the examined chilled *Tilapia nilotica* showed significantly greater *Pseudomonas* count than chilled *Mugil cephalus*, whereas frozen mackerel was significantly higher than the frozen saurus samples (Table 2).

Table 2: Statically analytical results of total *Pseudomonas* count (CFU/g) of the examined fish samples (n=30). Various letters indicated a statistically significant difference between the means at $p < 0.05$

Samples	Positive samples		Count CFU/g		
	No.	%	Min.	Max.	Mean ± SE
Frozen Mackerel	19	63.3	$2.9x10^2$	$3.5x10^5$	$8x10^{4a}±2.5x10^4$
Frozen Saurus	17	56.6	$4.7x10^2$	$1.3x10^4$	$5x10^{3b}±1.1x10^3$
Chilled *Tilapia nilotica*	20	66.6	$1.6x10^2$	$5.4x10^5$	$1x10^{5a}±3.8x10^4$
Chilled *Mugil cephalus*	22	73.3	$1.4x10^2$	$1.7x10^4$	$5x10^{3b}±1.1x10^3$

The isolated *Pseudomonas* spp. were identified into *Pseudomonas aeruginosa*, *Pseudomonas diminuta* and *Pseudomonas fluorescenes*. The results of the obtained data in our study, revealed that *Pseudomonas aeruginosa* was the highly isolated from Mackerel followed by saurus, *Mugil cephalus* and *Tilapia nilotica*. *Pseudomonas fluorescenes* and *Pseudomonas diminuta* were also isolated and identified with various percentages. However, some species couldn't be identified by the available biochemical tools (Table 3).

Table 3: Incidence of isolated *Pseudomonas* species in the examined fish samples (n=30)

***Pseudomonas* spp.**	**Frozen fish**				**Chilled fish**			
	Mackerel		**Saurus**		**T. nilotica**		**M. cephalus**	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Pseudomonas aeruginosa	10	33.3	9	30	7	23.3	8	26.6
Pseudomonas diminuta	3	10	3	10	2	6.6	5	16.6
Pseudomonas fluorescenes	4	13.3	3	10	2	6.6	1	3.3
Unidentified species	2	6.6	2	6.6	9	30	8	26.6

Furthermore, the psychrotrophic bacterial count was conducted. The results showed that, the frozen mackerel samples showed significantly high contamination level with a mean value $1.3x10^6$ CFU/g, while chilled *Mugil cephalus* had the lowest count (Table 4).

Table 4: Statically analytical results of total psychrotrophic bacterial count (CFU/g) of examined fish samples (n=30). Various letters indicated a statistically significant difference.

Samples	**Count CFU/g**		
	Minimum	**Maximum**	**Mean ± SE**
Frozen Mackerel	$6.6x10^3$	$7.1x10^6$	$1x10^{6a}±4.1x10^5$
Frozen Saurus	$1.2x10^3$	$6.7x10^5$	$1x10^{5b}±4.1x10^4$
Chilled *Tilapia nilotica*	$2.4x10^3$	$3.5x10^6$	$8x10^{5a}±2.2x10^5$
Chilled *Mugil cephalus*	$1.1x10^3$	$2.3x10^5$	$7x10^{4c}±1.5x10^4$

The Predominant Gram-positive bacteria have been identified among psychrotrophic bacteria. The result showed the occurrence of *Staphylococcus* spp. with high level followed by *Micrococcus* spp. whereas *Bacillus* spp. were identified by low incidence. Furthermore, Gram-negative bacteria, such as *Aeromonas* spp. *and Achromobacter* spp. were identified with various contamination level as displayed (Table 5).

Table 5: Incidence of identified psychrotrophic bacteria isolated from examined fish samples (n=30).

Microorganisms	Frozen fish				Chilled fish			
	Mackerel		Saurus		*Tilapia nilotica*		*Mugil cephalus*	
	No.	%	No.	%	No.	%	No.	%
Gram-positive bacteria								
Staphylococcus spp.	10	33.3	3	10	11	36.6	12	40
Micrococcus spp.	5	16.6	4	13.3	5	16.6	11	36.6
Bacillus spp.	2	6.6	0	0	3	10	1	3.3
Gram-negative bacteria								
Aeromonas spp.	5	16.6	7	23.3	5	16.6	6	20
Achromobacter spp.	8	26.6	11	36.6	0	0	11	36.6

Molecular detection of virulence genes.

Some isolates among *Pseudomonas aeruginosa* were selected for further study on the occurrence of *oprL*, *phzM and toxA* virulence genes. The results showed that all selected isolates harbored *oprL*, *phzM* and *toxA* virulence genes as shown in Fig.1, Fig.2 and Fig.3, respectively.

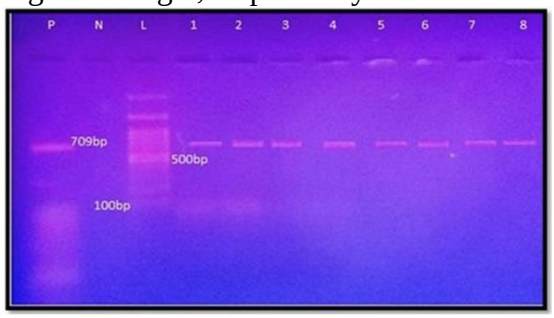

Fig. 1. DNA products from PCR reaction of amplification *oprL* gene in *Pseudomonas aeruginosa*. A PCR amplicons corresponding to 709 bp were obtained. P: positive control; N: negative control, L: ladder; lan1-lan8: +ve isolates.

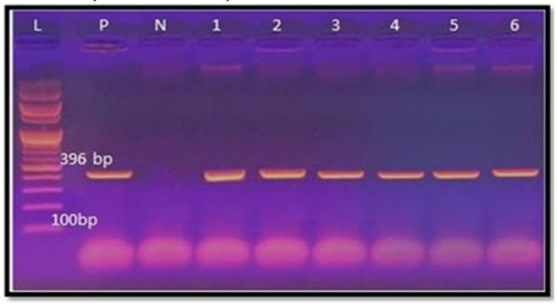

Fig. 2. DNA products from PCR reaction of amplification *toxA* gene in *Pseudomonas aeruginosa*. A PCR amplicons corresponding to 396 bp were obtained. L: ladder; P: positive control; N: negative control; lan1-lan6: +ve isolates.

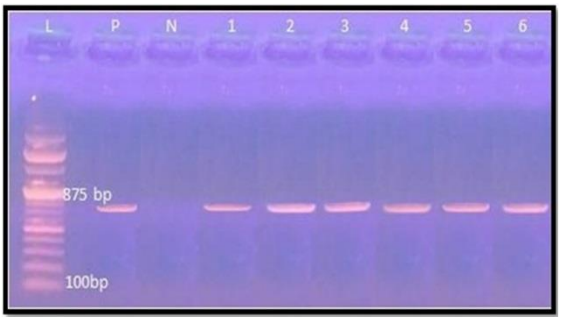

Fig. 3. DNA products from PCR reaction of amplification *phzM* gene in *Pseudomonas aeruginosa*. A PCR amplicons corresponding to 875 bp were obtained. L: ladder; P: positive control; N: negative control; lan1-lan6: positive isolates.

Discussion:

Bacteriological assessment of fish for the occurrence of *Pseudomonas* spp. has considerable significance as they are markers of meat quality as well, they may induce foodborne illness. In the current work, a total of 120 fish samples, including

frozen Mackerel, frozen Saurus, chilled *Mugil Cephalus* and chilled *Tilapia nilotica* (30 of each) were collected from various shops and markets at Sohag governorate, Egypt, for evaluation the existence of *Pseudomonas* spp. the results showed that 65% (78/120) samples were contaminated with *Pseudomonas* spp. Our findings were lower than those obtained by Abd El-Aziz (2015), who reported that all examined fish samples collected from Assiut city, Egypt was contaminated with *Pseudomonas* spp. Duman et al (2021) isolated ninety *Pseudomonas* strains from fish farms in Turkey and classified into 12 species and seven new *Pseudomonas* species were reported.

The obtained data in our study revealed that the highest *Pseudomonas* counts was Mackerel followed by Saurus in frozen fish samples, while in fresh chilled fish samples were *Tilapia nilotica* followed by *Mugil cephalus* with significant difference. The variation in the results between different species may be due to the difference in hygiene measures applied during catching, handling, freezing, storage and method of thawing in fish as mentioned by Salem et al. (2018).

The occurrence of *Pseudomonas aeruginosa* in the examined samples may be attributed to the fact that this specie commonly found in human, animals, plants and are a zoonotic important. Lower results were obtained by Benie et al. (2016), Ibrahim et al. (2016) and Salem et al. (2018).

Notably, mackerel samples showed the highest contamination level with psychrotrophic bacteria. These results may attribute to that the Mackerel contains high amount of fat and oils that favour growth of bacteria (Salem et al., 2018). The psychrotrophic count in *Saurus spp.* came in accordance with those reported by El-Shafey (2014) and higher results obtained by El-Sayed (2016) and El-Noby (2002) in frozen *Mugile Cephalus* and Mackerel. However, lower results were obtained by Salem et al. (2018)

Furthermore, psychrotrophic bacteria were differentiated into Gram- positive bacteria including *Staphylococcus* spp., *Micrococcus* spp. and *Bacillus* spp. as well Gram-negative bacteria were identified including *Aeromonas* spp. and Achromobacter spp. Nearly similar results were obtained by El-Hady and Samy (2011); Bahurmiz et al. (2016) and El-Sayed (2016).

In the present work, the PCR results showed that all selected isolates were positive for *oprL, phzM* and *toxA* virulence genes. Similar result was reported by Khattab et al (2015). L- lipoproteins mean the outer membrane protein associated with *Pseudomonas* aeruginosa that allow the bacterium to withstand the disinfectant and antibiotics. *oprL* is limited to *Pseudomonads*, so it could be a trustworthy maker utilized in recognition and pathogenicity assessment (Remans et al., 2010). Exotoxin A is an extracellular protein of pathogenic *Pseudomonas aeruginosa* and works by prevention of protein-synthesis in the cell (Aljebory et al., 2018). Furthermore, the presence of *phzM* in studied strains suggested their capability to secret a phenazine toxin, which improves their existence and establishment in inverse conditions (Bradbury et al., 2010; Cezairliyan et al., 2013).

In brief, *Pseudomonas aeruginosa* is one of the main emerging pathogens usually recovered from fish. The current work has shown that fish sold in Sohag governorate, Egypt was contaminated with *Pseudomonas* species. Enhancing the hygienic status of the fish preparation areas and the necessity for proper sanitation measures among fish staffs are required.

Conflict of interest statement

The authors declare that there are no conflicts of interest regarding publication of this article.

Ethical Approval

The animal experimental protocols were approved by the Animal Care and Use Committee of Animal Health Institute, Doki, Giza, Egypt and by the Animal Care and Use Committee of South Valley University, Egypt.

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