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Original Article

Percentage Incidences of Bacteria in Mahseer (*Tor putitora*), Silver carp (*Hypophthalmichthys molitrix*) Fish Collected from Hatcheries and Local Markets of District Malakand and Peshawar of Khyber Pakhtunkhwa, Pakistan

Incidências percentuais de bactérias em Mahseer (*Tor putitora*), carpa-prateada (*Hypophthalmichthys molitrix*), peixes coletados em incubatórios e mercados locais do distrito de Malakand e Peshawar de Khyber Pakhtunkhwa, Paquistão.

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Abstract

Fish is the main source of animal protein for human diet. The aim of this study was to find out prevalence of pathogenic bacteria of two selected economically important fish of Pakistan namely Mahseer (*Tor putitora*) and Silver carp (*Hypophthalmichthys molitrix*). Live fish samples from hatcheries and dead fish samples from different markets of study area were randomly collected. The fish samples were analyzed for isolation, identification and prevalence of bacteria. The isolated bacteria from study fish were identified through biochemical test and about 10 species of pathogenic bacteria were identified including the pathogenic bacteria to human and fish namely, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus iniae*, *Serratia spp. Citrobacter spp. Stenotrophomonas spp. Bacillus spp.* and *Salmonella spp.* The bacterial percentage frequency of occurrence in Silver carp and Mahseer fish showed *Pseudomonas aeruginosa* 21.42%, *Staphylococcus epidermidis* 17.85%, *Escherichia coli* 11.90%, *Staphylococcus aureus* 9.52%, *Citrobacter spp.* 9.52%, *Serratia spp.* 8.33%, *Streptococcus iniae* 7.14%, *Stenotrophomonas spp.* 5.95%, *Bacillus spp.* 4.76% and *Salmonella spp.* 3.57%. The study revealed that Fish samples of Mahseer and Silver carp that were collected from markets have found more isolates (10 bacterial species) than did the fresh fish pond samples (03 bacterial species) of hatcheries. The occurrence of pathogenic bacteria in study fish showed risk factor for public health consumers.

Keywords: prevalence %, bacterial species, Mahseer fish, silver carp fish, biochemical characterization.

Resumo

O peixe é a principal fonte de proteína animal para a alimentação humana. O objetivo deste estudo foi descobrir a prevalência de bactérias patogênicas de dois peixes economicamente importantes selecionados do Paquistão, nomeadamente Mahseer (Tor putitora) e carpa prateada (Hypophthalmichthys molitrix). Amostras de peixes vivos de incubatórios e amostras de peixes mortos de diferentes mercados da área de estudo foram coletadas aleatoriamente. As amostras de peixes foram analisadas quanto ao isolamento, identificação e prevalência de bactérias. As bactérias isoladas dos peixes do estudo foram identificadas através de testes bioquímicos e cerca de 10 espécies de bactérias patogênicas foram identificadas incluindo as bactérias patogênicas para humanos e peixes, nomeadamente, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus iniae, Serratia spp. Citrobacter spp. Stenotrophomonas spp. Bacillus spp. e Salmonella spp. A porcentagem de freqüência de ocorrência bacteriana em carpa prateada e peixes Mahseer mostrou Pseudomonas aeruginosa 21,42%, Staphylococcus epidermidis 17,85%, Escherichia coli 11,90%, Staphylococcus aureus 9,52%, Citrobacter spp. 9,52%, Serratia spp. 8,33%, Streptococcus iniae 7,14%, Stenotrophomonas spp. 5,95%, Bacillus spp. 4,76% e Salmonella spp. 3,57%. O estudo revelou que as amostras de peixes de Mahseer e carpa prateada coletadas nos mercados encontraram mais isolados (10 espécies bacterianas) do que as amostras de peixes frescos (03 espécies bacterianas) de incubatórios. A ocorrência de bactérias patogênicas nos peixes do estudo apresentou fator de risco para consumidores de saúde pública.

Palavras-chave: % de prevalência, espécies bacterianas, peixe Mahseer, carpa-prateada, caracterização bioquímica.

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1. Introduction

Fish is the best source of proteins, minerals, vitamins and essential nutrients that are a supplementary requirement in diet for infants as well as adults (Abdullah et al., 2011). Fish contain essential amino acids, low cholesterol and polyunsaturated fatty acids (Adedeji et al., 2011). Among the successfully cultured fish, the Mahseer (*Tor putitora*) and Silver carp (*Hypophthalmichthys molitrix*) are economically important fish species in Pakistan (Khan et al., 2011). Many methods are used to preserve fish such as cooling and freezing (Asiedu and Sanni, 2002). In cold or freezer storage many changes such as biochemical changes in lipids and proteins can occur (Latip et al., 2013). The pathogenic bacteria namely, *Pseudomonas spp. Salmonella spp. Vibrio spp. Streptococcus spp. Bacillus spp. Edwardsiella spp. Flavobacterium spp. Corynebacterium spp. Pasteurella spp. Yersinia spp. Shigella spp. E. coli*, *Proteus spp. Staphylococcus spp. Serratia spp. Citrobacter spp. Aeromonas spp. Enterobacter spp. Micrococcus spp. Campylobacter spp. Klebsiella spp. Bacteroides spp.* and *Hafnia spp.* were identified in Tilapia fish (*Oreochromis niloticus*) tissues collected from ponds (Ampofo and Clerk, 2010). Prevalences of bacteria namely *Bacillus cereus, Streptococcus pyogenes, Serratia odorifera, Enterobacter amnigenus, Pseudomonas aeruginosa, Salmonella typhimurium* and *Shigella flexneri* were detected in Rainbow trout (*Oncorhynchus mykiss*) fish collected from different trout hatcheries (Kousar et al., 2020). The bacterial isolates such as *Bacillus moratorium*, *Bacillus pumilus*, *Bacillus licheniformis*, *Listeria monocytogenes*, *Providential stuartii*, *Serratia marcescens*, *Salmonella spp.* and *Staphylococcus saprophyticus* were identified in collected Tilapia fish (*Oreochromis niloticus*) from different markets (Shinkafi and Ukwaja, 2010). When bacteria attached to any organ of fish at that time are harmless, it becomes harmful after multiplication and production of enzymes inside fish (Zaman et al., 2011). Whenever the human consumed infected fish with pathogenic bacteria it causes serious diseases such as *Vibrio cholera*e causes cholera, *Salmonella spp.* causes salmonellosis, *Shigella dysentariae, Shigella flexneri, Shigella boydii* and *Shigella sonnei* causes shigellosis, while *Mycobacterium tuberculosis* causes TB and *Escherichia coli* causes dysentery (Okafor, 1985; Austin and Austin, 2007).

2. Objectives

The objectives of our research were to find out prevalence of pathogenic bacteria and its percentage incidences in selected economically important fish namely Mahseer (*Tor putitora*) and Silver carp (*Hypophthalmichthys molitrix*).

2. Materials and Methods

2.1. Ethical statement

This experiment was approved by the ethical review committee of Abdul Wali Khan University Mardan, Pakistan and further the experimental fish were handled in accordance with the ethics of Society for the Prevention of Cruelty to Animals (SPCA) of Pakistan.

2.2. Area of study and collection of fish samples.

This study was carried out at Mahseer Fish Hatchery Thana Malakand and Carp Hatchery and Training center Peshawar of K.P.K. Pakistan from February, 2018 to December, 2019. For analysis of prevalence of pathogenic bacteria two species of fish i.e. the warm water cultural able Silver carp fish (*Hypophthalmichthys molitrix)* and semi cold-water culture able Mahseer fish (*Tor putitora*) were selected. Live fish samples (240) were collected from both hatcheries and dead fish samples (240) were collected from different markets of study area.

2.3. Storage and isolation of bacteria from fish samples in laboratory

After fish collection, immediately samples from skin, muscles and fins were taken by scraping sterilized cotton swab stick after that rubbed cotton swab stick was placed in sterile normal saline (0.87% Nacl). Each swab sticks tubes were labeled with name, place and date of collection. The swab sticks tubes were put on ice packed and transferred to laboratory immediately and were stored in refrigerator for further bacteriological analysis.

2.4. Bacteriological examination through biochemical characteristics test

Preparation of the media, identification and isolation of bacteria were carried out by applying method described by Cheesbrough (1984) and Olutiola et al. (1991).

2.4.1. Preparation of serial dilutions

In the test tube rack five separates sterilized test tube (10 ml volume) were arranged serially having 9 ml of distilled water for each sample. The contaminated scraped swab sticks were inserted in 1st test tube having 9 ml of distilled water and was considered as a stock. The 1 ml (1000µl) of sample from 1st test tube were picked by pipette and inserted to second test tube, then it was shake properly from 2nd test tube up to the 5th test tube were repeated respectively.

2.4.2. Inoculation to solid medium

After serial dilution process, 0.1 ml diluted samples were taken and was spread on nutrient agar after that it was incubated at 37°C for about 24 hours. Different media (MacConkey agar, Mannitol Salt Agar etc) were also used for specific bacterial isolation.

2.4.3. Gram staining and microscopic examination

From culture plate a colony was picked and then thin bacterial smear was prepared. Through Crystal violet it was stained for about one minute then it was rinsed with water. Few drops of Iodine solution were added for one minute then it was rinsed with water. The same smear was further washed for 10 to 30 second with decolorized agent i-e absolute ethanol after that again rinsed with water. Finally Safranin for 30 second to 1 minute was

added and rinsed with water. The slides were dried and were observed with light microscope.

2.4.4. Biochemical characteristics test

After gram staining and microscopic examinations different biochemical tests were performed for the identification of bacteria according to the methods as described by Olutiola et al. (1991), Cappuccino and Sherman (1996), Cowan and Steel (2002), Cheesbrough (2006) and Perilla (2003).

2.4.4.1. Catalase test

A drop of 3% H_2O_2 was added to center of clean slide. A colony was mixed with it. The result was interpreted for positive that is immediate bubbles formation and for negative as no gas formation.

Control microbes were used for the catalase test:

Staphylococcus spp. = +ve control and *Streptococcus spp.* = -ve control

2.4.4.2. Oxidase test

Oxidase reagent was added to clean filter paper. A colony was rubbed on it. The result was interpreted for positive and negative. Positive showed dark blue-purple color appearance in few second and negative showed no changes in color.

Control microbes were used for the oxidase test:

Pseudomonas aeruginosa = Positive control and *Escherichia coli* = Negative control

2.4.4.3. Simmon's Citrate Utilization Test

In test tubes of citrate media, slant were inoculated with suspected colony. After incubation for 24 hours at 37°C the result was interpreted for positive and negative. Positive showed dark blue color and negative turned to green in color.

Control microbes were used for Citrate test:

Klebsiella pneumoniae = Positive control and *Escherichia coli.* = Negative control

2.4.4.4. Sulfur Indole Motility Media (SIM) Test

SIM test tubes were inoculated with suspected colony by straight stabbing. After incubation for 24 hours at 37° C the result was interpreted.

For H2S Production: In positive result the media turned to black in color, while in negative no change in color was observed.

Control microbe: *Proteus mirabilis* is positive for H_2S production.

For Motility: The positive result showed movement from the line of stabbing and negative showed no motility.

Control microbes:

Pseudomonas aeruginosa and the strain of *Proteus mirabilis* are positive for motility.

For Indole: Few drops of Kovac´s reagent were added. In positive result red color ring formation at top of tube were noticed.

Control microbe: *E. coli* is positive for Indole.

2.4.4.5. Methyl Red / Voges-Proskauer (MR/VP) Test.

For Methyl Red: The suspected colony of bacteria was incubated for 24 hours at 37°C after that Methyl red reagent was added to MRVP broth. For positive result broth turned to red color and negative turned to yellow in color.

For Voges-Proskauer (VP): The suspected colony of bacteria was incubated in MRVP broth for 24 hours at 37°C after that few drops of 5% Alpha-naphthol or VP 1 reagent was added to MRVP broth. The broth was shacked then few drops of 40% KOH or VP 2 was added to the same broth. About 15 minutes waiting in broth red color in ring was noticed for positive result.

2.4.4.6. Urease test

In test tubes of urease media, slant were prepared and colony was inoculated on its surface. After incubation for 24 hours at 37°C the result was interpreted. In the positive test result the medium was changed to bright pink color, while in negative test the medium was changed to yellow color.

Control microbes were used for the urease test:

Proteus spp. = Positive control and *Escherichia coli* = Negative control.

3. Results

3.1. Identification of bacteria by biochemical test and prevalence % of the bacterial isolates

The most prevalent isolates bacteria that identified through biochemical test from study fish were *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus iniae*, *Serratia spp. Citrobacter spp. Stenotrophomonas spp. Bacillus spp.* and *Salmonella spp.* (Table 1). More bacterial isolates i-e 10 bacterial spp. were collected from market fish samples of Mahseer and Silver carp, while less bacterial isolates i-e 3 bacteria spp. were collected from fresh samples of Mahseer and Silver carp of hatcheries (Table 2). During this study, prevalence % of bacterial spp. showed *Pseudomonas aeruginosa* (21.42%), *Staphylococcus epidermidis* (17.85%), *Escherichia coli* (11.90%), *Staphylococcus aureus* (9.52%), *Citrobacter spps.* (9.52%), *Serratia spp.* (8.33%), *Streptococcus iniae* (7.14%), *Stenotrophomonas spp*, (5.95%), *Bacillus spp.* (4.76%) and *Salmonella spp.* (3.57%) respectively in collected fish samples (Table 3).

The study revealed that fresh ponds samples of fish showed good quality as compared to markets fish samples of study area. *Pseudomonas aeruginosa* showed highest occurrences (21.42%) followed by *Staphylococcus epidermidis* (17.85%) and *Escherichia coli* (11.90%), while *Salmonella spp.* showed lowest percentage (3.57%) occurrences (Figure 1).

4. Discussion

In present study the more bacterial isolates in markets fish samples as compared to fresh fish samples was recorded. The reason behind this might be that in live fish

Table 1. Biochemical test and Microscopy of bacteria species in collected fresh and market fish samples

Bacterial Isolates	Gram staining	Shape	Catalase	Oxidase	Methyl red	Voges proskauer	Citrate	Urease	SIM		
									H_2S	Indole	Motility
Pseudomonas aeruginosa	-	Rod	+	+	-	-	+	-	-	-	+
Escherichia coli	-	Rod	+	-	+	-	-	-	-	+	+
Staphylococcus aureus	+	Cocci	+	-	+	+	+	+	-	-	-
Staphylococcus epidermidis	+	Cocci	+	-	-	+	-	+	+	-	-
Streptococcus iniae	+	Cocci	-	-	N/A	-	-	-	-	-	-
Serratia spp.	_	Rod	+	-	-	+	+	+	_	-	+
Citrobacter spp.	-	Rod	+	-	+	-	+	+/-	+	-	+
Stenotrophomonas spp.	-	Rod	+	-	-	-	-	-	-	-	+
Bacillus spp.	+	Rod	+		+/-	-	+	+	N/A	-	+
Salmonella spp.	_	Rod	+	_	+	_	_	_	+	_	+

Key: - = Negative reaction, + = Positive reaction, N/A = Not applicable, SIM = Sulfur Indole Motility test, H_2S = Hydrogen sulfide.

Table 2. Bacterial isolates in selected fresh and markets fish samples.

Bacterial Isolates	Mahseer and Silver carp (Fresh fish)	Mahseer and Silver carp (Market fish)
Pseudomonas aeruginosa	+	+
Escherichia coli	-	+
Staphylococcus aureus	-	+
Staphylococcus epidermidis	-	+
Streptococcus iniae	-	+
Serratia spp.	-	+
Citrobacter spp.	+	+
Stenotrophomonas spp.	-	+
Bacillus spp.	-	+
Salmonella spp.	+	+

Key: - = Isolates absents + = Isolates presents

Table 3. Prevalence % of bacterial isolates in collected fresh and markets fish samples.

Bacterial Isolates	No. of Isolates	Prevalence %
Pseudomonas aeruginosa	18	21.42
Escherichia coli	10	11.90
Staphylococcus aureus	08	9.52
Staphylococcus epidermidis	15	17.85
Streptococcus iniae	06	7.14
Serratia spp.	07	8.33
Citrobacter spp.	08	9.52
Stenotrophomonas spp.	05	5.95
Bacillus spp.	04	4.76
Salmonella spp.	03	3.57
Total Isolates	84	100

their own immunity system can prevent it from bacterial spoilage. As mentioned by Abolagba et al. (2011) that the defense system of a fish becomes collapse after the death of fish which is further attack by microbes. This is further supported by Nwiyi and Onyeabor (2012), that when fish is captured, it that time fish can be contaminated by various microorganisms through unhygienic practices such as use of contaminated equipments, rough handling and storage facilities.

The result obtained in our study indicated that in collected fish the pathogenic bacteria i-e *Escherichia coli* and *Salmonella spp.* etc were found in pond. The occurrences of some pathogenic bacteria in fresh samples might indicate that these bacteria came to pond from outside environments. As described by Jayasinghe and Rajakaruna (2005), that many bacteria such as *Salmonella, E.coli, V. cholera* and other coliforms comes from human and animal fecal matter to water and become polluted where fish survive.

The result of present study indicated occurrences of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus iniae*, *Serratia spp. Citrobacter spp. Stenotrophomonas spp. Bacillus spp.* and *Salmonella spp.* Similarly Shinkafi and Ukwaja (2010) reported bacteria in Tilapia fish (*Oreochromis niloticus*) that were collected from markets. The finding of pathogenic bacteria in this study might leads to zoonotic infection or intoxication in public health consumers.

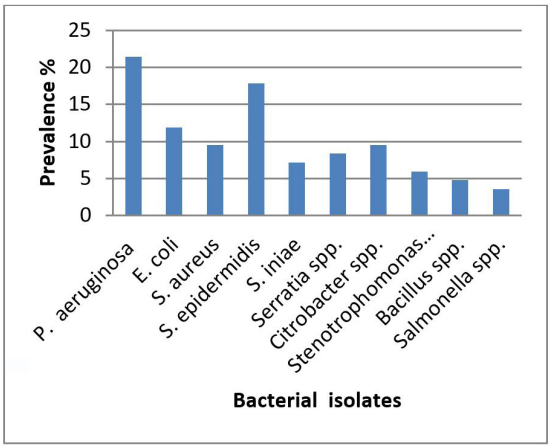

Figure 1. Prevalence % of bacterial isolates in collected fresh and markets fish samples.

As described by Han et al. (2001) that the presences of bacteria in a fish may cause human infection such as food poisoning and gastroenteritis by its consumption as undercooked or heated insufficiently.

5. Conclusion

1. The biochemical test result indicated that 4 species of positive bacteria namely *Staphylococcus aureus*, *Streptococcus iniae, Staphylococcus epidermidis*, *Bacillus spp.* and 6 species of negative bacteria namely *Pseudomonas aeruginosa, Escherichia coli, Serratia spp., Citrobacter spp., Stenotrophomonas spp., Bacillus spp.* and *Salmonella spp.* were identified in collected fish samples.
2. Fish samples of Mahseer and Silver carp that were collected from markets have found more isolates (10 bacterial species) than did the fresh fish pond samples (03 bacterial species).
3. *Pseudomonas aeruginosa* (21.42%) and *Staphylococcus epidermidis* (17.85%) were found more abundance in percentage incidences as compared to other bacteria.

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