Isolation and Identification of Pathogenic Bacterial Flora from Native Freshwater Fish of Kashmir Himalaya

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ARTICLE INFO	ABSTRACT
Article History: Received: Feb. 4, 2021 Accepted: Dec. 23, 2021 Online: Jan. 19, 2022 _____ **Keywords**: Bacteria, Identification, Indicator, *Schizothorax esocinus*, River Jhelum	Identification of freshwater fish's bacterial flora is an important tool for the determination of quality and the safety of human consumption. The aim of the present study was to isolate the bacterial flora of freshwater fish from gills, buccal cavity and skin surface of *Schizothorax esocinus*, from the locations of river Jhelum of Kashmir valley. A total number of 45 samples of freshwater fish were selected on the basis of clinical diagnosis. The fish sample was distributed on the basis of clinical inspection as gills [20%, (n=9)], skin [11.11%, (n=5)], gills and skin [24.44%, (n=11)], gills and buccal cavity [13.33%, (n=6)], skin and buccal cavity [8.90%, (n=4)], gills, skin and buccal cavity [22.22%, (n=10)], respectively. Statistically, the frequency distribution was not uniform and p<0.5. Freshwater fish's bacterial flora consists of *Pseudomonas aeruginosa, Staphylococcus* sp*., Enterococcus* sp*., Bacillus sp., Salmonella* sp*.* and *Escherichia coli.* The isolation of bacterial flora in fish serves as an indicator organism of anthropogenic pressures/activities of the aquatic environment. Their presence causes diseases for humans. The association of pathogenic bacterial species in fish may lead to food safety hazards for the consumers if the fish are handled or prepared improperly.

INTRODUCTION

Over the last 60 years, global fish production has significantly increased, reaching roughly 179 million tonnes in 2018 with a value of $401 billion **(FAO, 2020)**. Global fish consumption has also increased, from 9.0 kg per capita in 1961 to 20.5 kg in 2018. Globally, 88% of fish output is for human nutrition, with 44% in the form of live, fresh, or cold fish, the most desirable and expensive forms in many markets **(FAO, 2020)**. The remainder of the production is processed, with 35% frozen, 11% prepared and preserved,

and 10% dried, salted, smoked or otherwise cured (**FAO, 2020**). Because finfish account for more than 65% of total global food fish output, 76.5% of domestic landings in the US were consumed fresh and frozen, 1.9% canned, and 1.5% cured (**NOAA, 2020**). Global aquaculture production is expected to triple by 2050 due to the increased demand on high-quality protein, reduced wild fish harvest, and technological breakthroughs in fish farming (**Ibrahim *et al.,* 2020**).

India is globally the second largest producer of fish, as well as fresh water fish. With a total population of 2,80,63,537 fishmen (1,56,65,630 males and 1,23,97,907 females), fish production reached 141.64 lakh tonnes (37.27 lakh tonnes for marine and 104.37 lakh tonnes for inland fisheries) in 2019-20, up from 107.62 lakh tonnes (36 lakh tonnes for marine and 71.62 lakh tonnes for inland fisheries) in 2015-2016 (**Department of Fisheries, Govt. of India, 2020**). India's share of global fish production has steadily increased, from around 11.4% in 2016 to 13.5% in 2018 (**Department of Fisheries, Govt. of India, 2020**).

Fish are among the best sources of protein specified for human consumption; however, fish products may carry the spoilage, pathogenic and opportunistic pathogenic microorganisms, including the food borne pathogens (**FAO, 2000**). Fish consumers are vulnerable to risky effects caused by those pathogens (**Huss, 1997; Davies *et al.,* 2001; Rohde *et al.,* 2015**). Fishes engage in complex energy and material transferring processes in a wide range of trophic and symbiotic interactions, while they also serve as reservoirs and vectors of microorganisms (**Parris *et al.,* 2016; Ngugi *et al.,* 2017**). Such diversity makes fish communities useful for exploring the factors shaping animal associated microbiomes (**Lescak & Milligan-Myhre, 2017**). Gills are the primary spots for gas and waste exchanges in fish, adding to mucosal immune interactions, osmoregulation and detoxification (**Maina, 2002**). In addition, gill microflora may play significant roles in overall fish health and physiology.

Animal-associated microflora often contain hundreds of bacterial or archaeal species, many of which are hypothesized to contribute to host health, behavior and development (**Cantley & Clardy, 2015; Gilbert *et al.,* 2020**). Microbes, on the other hand, live in a variety of niches throughout the animal body, including associations with non-GI mucosal surfaces, which also participate in chemical or gas exchange as well as as barriers or entry points for pathogens. Characterization of non-GI mucosal microbiomes, such as those of the gills, lungs, and skin is therefore critical for identifying novel activities of animal-associated microflora and understanding the link between ambient and host microbial communities.

The mucosal surface of the gills of fish may also be a very unusual environment for host-associated bacteria. In contrast to the GI tract, where oxygen may be more limiting and the genetic capacity for anaerobic fermentative metabolism has been proven, the fish

gill is potentially influenced by high local concentrations of oxygen and nutrients **(Wilkie, 2002; Ngugi *et al.,* 2017)**. Furthermore, the gills may experience high levels of mucosal discharge, particularly during stressful conditions **(Hess *et al.,* 2015)**. Furthermore, gills may have high connectivity with the surrounding water microbiome as well as between the water and the bloodstream **(Maina, 2002)** and may act as crucial entry points for pathogens **(Hess *et al.,* 2015)**. Consequently, to make a decision regarding the safety of fish consumption, it is important to identify and characterize the fish bacterial flora. Fishing for consumption is very common in Jammu & Kashmir, but the microbiological assessment of freshwater fish caught in rivers and lakes have not yet been determined. Therefore, the aim of this study was to detect the bacterial flora of *Schizothorax esocinus* fish caught by the local fishermen.

MATERIALS AND METHODS

Chemicals, equipments and glassware

Nutrient agar (NA; supplemented with crystal violet and methyl red), Nutrient Broth medium, MacConky agar (MA) medium, High chrome medium, Blood agar Xylose lysine deoxycholate agar (XLD) medium, and Thiosulphate citrate-bile salts sucrose agar (TCBS) medium, glycerol, and ethanol were used in the present experiment. All chemicals and reagents were acquired from Merck and Sigma Aldrich (Germany), and Sigma Aldrich (Switzerland). 37ºC incubator, 37ºC shaker, hot air oven, laminar flow, analytical balance, digital weighing machine, Vortex shaker and steam sterilizer (autoclave) were all used.

Collection of fish samples

The current study estimates the bacterial flora of indigenous cold water species *Schizothorax esocinus* belonging to family Cyprinidae and order Cypriniformes. The samples of *S. esocinus* were collected from the locations of River Jhelum of Kashmir valley with the help of some fishermen and were identified by various taxonomic keys **(Tilak, 1987; Jhingram, 2007)**. After collection, the signs and symptoms related to infected fish were recorded. The fish samples were collected aseptically, labelled and immediately transported in a sterile container to the Advance Research laboratory and Parasitology laboratory, Department of Zoology, University of Kashmir - Hazratbal, Srinagar, Jammu and Kashmir. The sampled fish were then processed within 2- 4 hours.

Sampling

Swabs of skin and buccal cavity were taken for analysis. Each fish was swabbed from skin and buccal cavity and inoculated on Nutrient Agar, TSA, MacConkey agar and Blood agar **(Spanggaard *et al.,* 2000; Dar *et al.,* 2013; Dar *et al.,* 2015)**. Then, they

were incubated at 37ºC for 24 hours **(Eddy & Jones, 2002; Al-Harbi & Uddin, 2004, 2005)**.

The gills were cut with sterile scissors under aseptic condition and then processed individually (Fig. 1.). The gill samples were placed in 0.85% sodium chloride (saline water) solution, and 1gram of gill sample was crushed with the help of a homogenizer with about 10 ml of sterilized pre-chilled saline water, and the stock solution were serially diluted.

Fig. 1. Dissection of gill tissue

The gills homogenate was used as inoculum; samples were separately analyzed by ensuring homogeneity using a sterile pipette. An amount of 1 mL of each sample was suspended into 9 mL sterile saline water aseptically, and the outcome was then shaken together in a vortex shaker. Further serial dilution of 10-folds was carried out. An amount of 100µl of serially diluted homogenized samples were spread on duplicate sterilized culture media Nutrient Agar, TSA, MacConkey agar and Blood agar plates and incubated at 37ºC for 24 hours. Colonies with different morphological appearances were isolated. Purity of the isolates were determined by streaking separately on culture media plates. Cultures were maintained and stored at 4°C **(Ghosh *et al.*, 2002)**.

Identification of bacteria

After isolation, phenotypic characteristics (colonial morphology, microscopic appearance), staining technique (Gram's Staining), and biochemical tests were performed for the identification of available bacterial identification keys described in the studies of **Cruickshank *et al.* (1979)**, **Collins *et al.* (1994)**, **Cheesbrough (2006)** and **Mondal *et al.* (2010)**.

Pure cultures of the isolates were identified using biochemical characterisation using the criteria outlined in Bergey's Manual of Determinative Bacteriology **(Garrity, 2001)**. In addition, the analytical profile index of the API20-E system was used to confirm each strain **(Buller, 2004)**.

RESULTS

Sign and symptoms of infected fish

Fish samples were collected from the locations of River Jhelum in Kashmir valley. Some signs and symptoms were observed, such as hemorrhaging of skin and internal organs, bulging eyes, swollen abdomens, reddish tints/spots to eyes, gills, skin, and fins and abnormal behavior, respectively.

Isolation and identification of bacteria

Table (1) represents the number of fish samples per area, from where the isolation of the bacterial species were taken, including gills [20%, (n=9)], skin [11.11%, (n=5)], gills and skin [24.44%, (n=11)], gills and buccal cavity [13.33%, (n=6)], skin and buccal cavity [8.90%, (n=4)], gills, skin and buccal cavity [22.22%, (n=10)], respectively. Statistically, the frequency distribution was not uniform and $p<0.5$.

The various types of selective, enriched and differential media were used for the isolation of pure colonies of bacterial species. Methyl red and crystal violet indicators were practiced for the screening of Gram-negative bacteria in comparison to Gram-positive bacteria (Fig. 2). It was witnessed that all isolated species showed growth on types of medium used. Five bacteria species were isolated and identified, such as *Staphylococcus* sp.*, Enterobacter* sp.*, Pseudomonas* sp.*, Bacillus* sp., *Escherichia coli* and *Salmonella* sp. (Table 2 & Fig. 3).

A   B
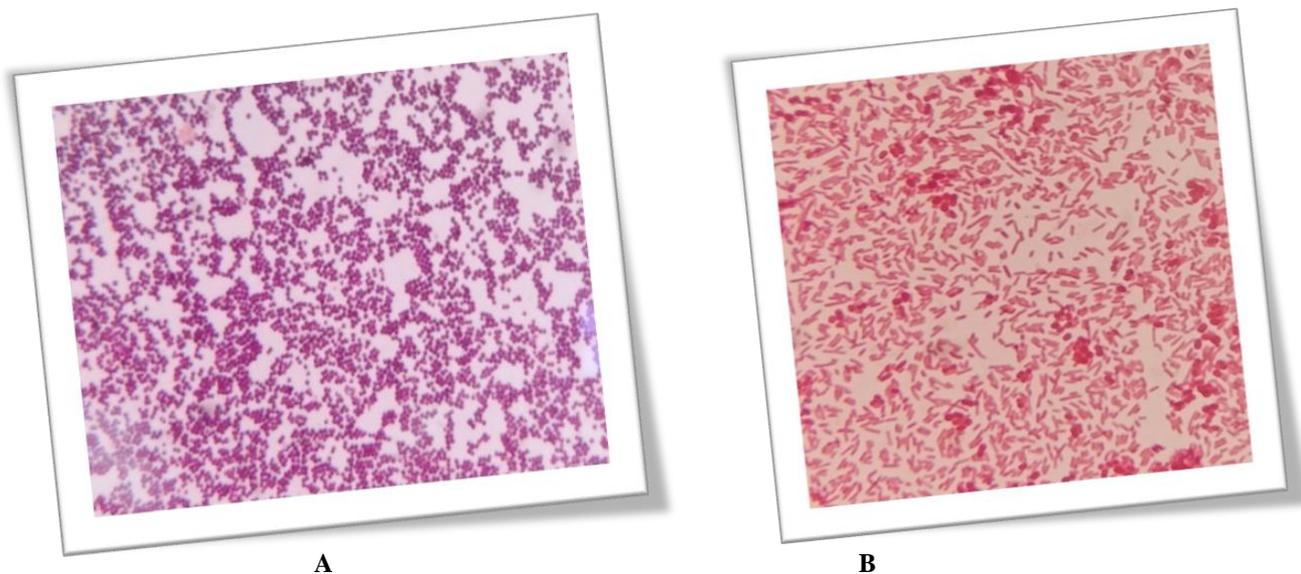
Fig. 2. (A) Gram- positive *cocci* and **(B)** Gram- negative *bacilli*

Escherichia coli

Salmonella sp.

Pseudomonas sp.

Enterobacter sp.

Bacillus sp.

Staphylococcus spp.

Fig. 3. Pure culture of bacterial species on different media

Table 1. The percentage distribution of fish samples on basis of diagnosis

Area	Frequency (N)	Percentage (%)	Chi Square	*P*-value
Gills	9	20.00		
Skin	5	11.11		
Gills, Skin	11	24.44		
Gills, Buccal Cavity	6	13.33	**5.533**	**p<0.5**
Skin, Buccal Cavity	4	8.90		
Gills, Skin, Buccal Cavity	10	22.22		
Total	**45**	**100**		

Table 2. Biochemical tests for the identification of fish associated bacterial flora
GNB= Gram negative bacilli, GPC= Gram positive cocci; (+) = Positive; (-) = Negative.

Biochemical Tests	*Enterobacter* sp.	*Escherichia coli*	*Pseudomonas Aeruginosa*	*Bacillus* sp.	*Salmonella* sp.	*Staphylococcus* spp.
Gram Reaction	**GNB**	**GNB**	**GNB**	**GPC**	**GNB**	**GPC**
Catalase test	+	+	+	+	+	+
Citrate test	+	-	+	+	+	-
Indole test	-	+	-	-	-	-
Methyl red Test	-	+	-	-	+	-
Oxidase test	-	+	+	-	-	-
Urease test	-	-	+	-	-	+
Motility test	+	+	+	+	+	-
Carbohydrate Test	+	+	+	+	+	+
Voges proskeur test	+	-	-	-	-	+

DISCUSSION

Microflora is a significant part of the aquatic ecosystem, and the interaction between the pathogenic microflora and this aquatic environment result in the contagious diseases in aquaculture methods **(Noga, 2000; Ikpi & Offem, 2011)**. The occurrence of the pathogenic bacterial species cause the contamination of the aquatic water bodies originated from animal wastes **(Abdelhamid *et al.,* 2006)**. The isolation of *E. coli, shigella* and *salmonella* from the fishes indicates the presence of contamination by fecal matter, which pollutes the environment **(Yagoub *et al.,* 2009)**. The prevalence of *Staphylococcus aureus* has public health significance for it has been incriminated with food-borne intoxication and infection. Its ability to produce enzymes and toxins is responsible for this effect **(Umoh & Odoba, 1999)**.

The aquaculture sectors are prone to several diseases, increasing the vulnerability to pathogenic infections **(El -Sayed, 2006)**. In addition, the dispersion of mobile Aeromonas spp. in the aquatic environment and fish has been documented **(Kaper *et al.,* 1981; Abeyta *et al.,* 1986; Hatha *et al.,* 2005; Hussain *et al.,* 2014)**. Similarly, **Noor El Deen *et al.* (2014)** discovered that skin ulcers could be the source of infection produced by Aeromonas spp. The findings are consistent with those of **Hazen *et al.* (2012)** who suggested that, the colour of a bacterial colony may be utilized to establish its genus level. Aeromonas spp. is an opportunistic pathogen that can be found in freshwater habitats around the world, as well as in soil, water and food. This bacterium can cause foodborne and nosocomial diseases **(Cabral, 2010; Janda, 2010; Gauthier, 2015)**.

In this study, five bacteria species were detected from the gills, skin, and buccal cavity of *S. esocinus.* Among the isolated bacteria, six bacterial species were identified, such as *Staphylococcus* sp*., Enterobacter* sp*., Pseudomonas aeruginosa, Bacillus* sp*. Escherichia coli* and *Salmonella* sp. This result coincides with the findings of **Ahmed and Naim (2002)** who postulated that, bacteria isolated from brackish pond water, sediments, and healthy tilapia farmed in Saudi Arabia were largely Gram-negative; a result that matches with the present findings. Some marine fish species showed septicemic bacterial infections, including *vibrios, aeromonads, pseudomonads, photobacteria, streptococci*, and *staphylococci* during various stages of development **(Alicia *et al.,* 2005; Samuelsson *et al.,* 2006)**.

The organism may contaminate the smoked fish through human handlers. Its survival may be related to its ability to survive at high salt levels. It can grow well at temperatures of 30- 37ºC **(Brown, 2004)**. The presence of *Bacillus* and *Salmonella* species are also important, since they may result in food borne illnesses. The presence of *Salmonella* sp. indicates poor food preparation and handling practices such as cross contamination. Most of the isolated organisms are probably contaminants, specifically after the smoking operation, and they are mostly spoilage organisms.

Nevertheless, potentially pathogenic organisms, isolated from smoked fish, showed the need for proper hygienic conditions for the processing and distribution of products. **Brown (2004)** noted that the selling of smoked fish on wooden or metal tables on open quay-sides increases the several potential bacteriological dangers, and the more or less lengthy the exposure to increasing temperature is, the more spoilage bacteria are permitted to multiply.

The presence of the enteric bacteria in fishes serves as an indicator of fecal contamination and water pollution. The association of pathogenic bacteria in fish suggested that, if fish were not properly handled or prepared, food safety with respect to consumers would be prone to hazards **(Akila & Kumaran, 2018)**. During this study, the fish samples sheltered human disease-causing organisms, among which food poisoning, diarrhea, typhoid, fever etc are considered. The micro-organisms such as *Pseudomonas, Salmonella, S. aureus* and *shigella* are present in food material, causing most foodborne diseases, considering the environment and consumers. **Sujatha *et al.* (2011)** reported the same type of human pathogenic bacteria in two edible fishes, *Priacanthus hamrur* and *Megalaspis cordyla* at Royapuram of Chennai, India. Moreover, **Akila and Kumaran (2018)** reported the same types of pathogenic species during their study, which are very harmful to the human population.

CONCLUSION

The present study concluded that water of the River Jhelum was contaminated due the anthropogenic activities. Different bacterial strains were isolated from a fish association, which exhibit satisfactory microbiological quality. These bacteria cause a serious threat to fishes and their consumers as well. The *Pseudomonas* sp. and *Staphylococcus* sp. were mostly present, causing rapid fish deterioration. The dominance of this group indicates that the fish are prone to spoilage processes.

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