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Isolation and antimicrobial profiling of pathogenic bacteria from Fish samples

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Manuscript details:	ABSTRACT

Fish is important nutrient source of protein because it contains more than 60% of protein source. The advantage of fish as a food is that it has easy digestibility and nutritional value. The range of bacterial genera isolated which are related to the aquatic habitat of fish. These include the change in flora during storage. Some disease-causing pathogenic bacteria grow on fish which can survive in freezing temperature and will resume growth when thawing. The bacterial species that are facultative pathogenic for fish and human being and may be isolated from fish without apparent symptoms of the disease. In assessment to isolate and identified these pathogenic bacteria from different variety of fish parts such as Skin, Gills, Intestine, Buccal cavity and study their trends of susceptibility and resistant pattern with various antibiotic, the present work was undertaken. In this study pathogenic bacterium such as S. typhi, S. aureus, P. aeruginosa, E. coli Were isolated. This study demonstrates that fish is potential source of infectious diseases. In antibiotic resistant E. coli and S. typhi was more resistant to gentamycin, and ampicillin. It was also found that S. aureus more resist to gentamycin, ciprofloxacin as compared to amoxicillin, erythromycin, and chloramphenicol.

Keywords: Pathogenic Bacteria from Fish, Skin, Gills, Intestine, Buccal cavity, *S. typhi, S. aureus, P. aeruginosa, E. coli*

INTRODUCTION

Fish is important nutrient source of protein because it contains more than 60% protein source. The advantage of fish as food is easily digestibility and nutritional value (Sichewo *et al*., 2013). Aquaculture sector is the biggest and fastest fish food growing sector as animal production. Total fisheries production in 1950 to 2004 was increases up to 1 million ton to 141 million ton (Silva *et al*., 2010). In India, oil sardine is one of the most major resources of our country. About 20-25 % all Indian covered by aquaculture fisheries. Large scale of oil sardine has been observed in kerala and Karnataka. The difference fishes represent the greatest taxonomic and Ecological diversity of vertebrate (Clements *et al*., 2014).

Fish contains higher number of bacteria associated with the skin (<50 to 0.30×10^5), (<10 to 1.4×10 /gills) & (7.5×10^6 x 8.5 x 10^8/intestine) but some fishes grown inside the waste pond or in a natural freshwater environment (Cahill,1990).

The microbial diversity of fresh fish muscle depends on the fishing ground as well as environment factor around it. Fish containing pathogenic bacteria associated with fish as indigenous and non-indigenous. The non-indigenous contaminated the fish or the habitat one way for example *E.coli, Cl. botullinum, Shigella dysentery, S. aureus, Salmonella* and *L. monocytes.* The indigenous bacterial pathogens are found naturally living in the fish habitat for example *Vibrio, Aromonas spp* (Sichewo *et al.,* 2013).

Bacterial population are associated with reared marine fish are important to nutrition as furnish cell substances or micronutrient such as fatty acid, vitamin, mineral or some enzyme (Blanch *et al.,*2008). Bacterial flora isolated from the skin, gills, intestine, B. cavity etc. have been described for a limited number of fish species. Generally, the range of bacteria genera isolated is related to the aquatic habitat of fish. The normal microbial flora associated with fish has been studied. These include the change in flora during storage. The mucus of fish gills, gut and skin contain lysozyme and immunoglobulin which act as defense mechanisms against bacteria. *Vibrio spp.* are isolated from fish only caught at marine sites, as well as *Aeromonas spp.* are present in highest number on gills of fish from upstream water. The number aerobic heterotropic bacteria found in digestive tract and anaerobes are detect only in upper intestinal tract (Cahill *et al.*, 1990).

Microbial food spoilage is an area of global concern it has been estimated that up to 25% of all food production is lost post-harvest owing to microbial activity. Day by day base on science understanding of growth and activity of spoilage microorganisms in seafood and other food is play very crucial role for development for the preservation technique and subsequent reduction of loss due to spoilage (Gram and Dalgaard, 2002).

Some disease causing pathogenic bacteria grown on fish like *Pseudomonas spp, Alcaligenes spp, Flavobacterium spp.* can survive in freezing temperature will resume growth when thawing (Eze *et al.*, 2011). The bacterial species that are facultative pathogenic for fish and human being and may be isolated from fish without apparent symptoms of the disease (Sichewo et al.*,* 2013).

Fish disease is the most harmful problem to the fish industries. Day by day increase the aquaculture activity several bacterial disease cause to the fish and then increase the rate of mortality and decrease the productivity. Some pathogenic bacteria on fish like *Flavobacterium, P. flurosence, V. cholrae, Aeromonas hydropila, P. aeruginosa, Enterococcus, S. aureus, Micrococcus* are commonly found bacterial agents on fish diseases (Sharma and shiag, 2013).

Fish and fish product are 3rd highest source of diseases (Shin *et al.,* 2004). *Enterobactericace* family group bacteria contaminated food and food product is worldwide problem (Ribeiro *et al.*, 2015). The greatest risk to the human health due to consumption of raw fish product from fish, because 55% micro-organisms are isolated from raw fish (Yogoub, 2009).

Fishes are often associated with human diseases. The zoonotic diseases associated with fish contact are primarily bacterial infections. Thus, it is obligate to study the prevalence of bacterial pathogens in fish to ensure the safety of fish products and environments. The bacterial assessment of fish also gives additional information about the hygienic status of environments, including lakes, rivers, ponds, and fish farms. Detection of pathogenic bacteria could be an important indicator of possible contamination. The aim of this research was to describe most relevant bacteria linked to fishes causing human food borne diseases.

MATERIAL AND METHODS

The present work was conducted from 2019 to 2020. In assessment to isolated and identified pathogenic bacteria from fish and study their susceptibility and resistant pattern with various antibiotics. Various pathogenic bacteria affect the different fishes as well as to reduce the quality of fish product and infected fish product to cause the disease for human being viz. Illness, food born disease.

Collection of Sample:
Different fish samples were collected from fish market of Akola city, such as Skin, Intestine, Buccal cavity, Gills. Those samples were transfer immediately in laboratory for further processing.

Isolation and identification of pathogenic bacteria by phenotypic method.

- *Propagation of sample:*

Sample were transferred in 0.5 ml of nutrient broth for enrichment and incubation for 37 ^{0} C for 24 hrs. Cultural media support the growth of bacteria.

- *Plating of Enrichment sample:*

All the samples after enrichment were compared with control tube and then inoculated on nutrient media. After inoculation, all the plates were incubated at 37^{0}C for 24 hrs.

Maintenance and preservation of cultural:
Organisms grown on appropriate media for 18 hrs were preserved on nutrient agar slant at 4^0 c in refrigerator and this culture used for routine laboratory works.

Morphological characterization:
As per the standard literature (Bergey's manual of systematic bacteriology, 2nd edition 1984), next day all typical colonies on nutrient media were screened for colony characterization and examined microscopically for Grams staining method.

Biochemical characterization:
All the pathogenic bacteria were then subjected for conventional Biochemical analysis (Bergey manual of systematic bacteriology, 2nd edition, 1984; Dubey and Maheswahri, 2006).

Cultural characterization (plating on selective media):
Colonies with relevant pathogen with different morphological character and Biochemical characters were selected to analyzed their cultural properties and inoculated on respective selective media viz. Mannitol salt agar, bismuth sulfite agar, XLD, MacConkey's agar blood agar, Pseudomonas isolation agar, etc. All the plate incubated in incubator for 37^{0}C for 24 hrs.

Antibiotic susceptibility testing:
After phenotypic characterization, the pathogenic bacteria were subjected for antibiogram. The antimicrobial susceptibility testing was done by the agar Disk Diffusion method as described by NCCLS 2002, and Kirby Bauer Disk Diffusion method, now known as the clinical and laboratory Standard institute (CLSI) (Bauer *et al.,*1966; CLSI, 2015).

Table No. 1: Antibiotics and their concentration

Antibiotics name	Concentration
Gentamycin	GEN10
Erythromycin	E 10
Chloramphincol	C30
Ciphrofloxin	CIP5
Amphicillin	AMP10
Amoxiclave	AMC30

Table 2: Frequency distribution.

Sr. No.	Isolated organisms	No. of isolates	% of isolates
1.	*Escherichia coli*	15	24.59
2.	*Staphylococcus aureus*	13	21.31
3.	*Salmonella typhi*	15	24.59
4.	*Pseudomonas aeruginosa*	18	29.50

Table 3: Morphological characterization

Sr. No.	characters	E. coli	S. aureus	S. typhi	P. aeruginosa
1.	Size	2mm	4mm	2mm	2mm
2.	Shape	Circular	Circular	Circular	Circular
3.	Margin	Regular	Regular	Regular	Regular
4.	Elevation	Convex	Convex	Convex	Convex
5.	Texture	Smooth	Smooth	Smooth	Smooth
6.	Opacity	Opaque	Opaque	Opaque	Opaque
7.	Color	Green metallic sheen	Yellowish	Red & black	Fluorescent color
9.	Grams characters	Short rod	Grapes like cluster	Short chain	Bacillus
10.	Motility	Motile	Non motile	Motile	Motile

Table 4: Result of sugar fermentation test isolated bacterial species from fishes.

Sr.No.	Name of sugar	Sugar fermentation	S. aureus	E. coli	S. typhi	P. aeruginosa
1.	Glucose	Acid/Gas	+ve	+ve	+ve	-ve
2.	Lactose	Acid/Gas	+ve	+ve	-ve	-ve
3.	Mannitol	Acid/Gas	+ve	+ve	+ve	+ve

Table 5: Result of IMViC test isolated bacterial species from fishes.

Sr.No.	Test of IMViC	S. aureus	E. coli	S. typhi	P. aeruginosa
1.	Indole	+ve	+ve	-ve	-ve
2.	Methyl red	+ve	+ve	+ve	-ve
3.	Vogesproskaur	+ve	-ve	-ve	-ve
4.	Citrate	+ve	-ve	-ve	+ve

Table 6: Enzyme Test**:** The use for identification of presence or absence of an enzymatic activity in particular type of microorganisms**.**

Sr.No	Enzymes	S. aureus	E. coli	S. typhi	P. aeruginosa
1.	Catalase	+ve	-ve	+ve	+ve
2.	Oxidase	-ve	-ve	+ve	+ve
3.	Coagulase	+ve	-ve	-ve	-ve
4.	Urease	+ve	-ve	-ve	-ve
5.	DNase	+ve	-ve	-ve	-ve
6.	Hemolysis	Beta	Beta	Beta	Beta

Table 7: Cultural characteristics

Sr. No.	Name of media	S.aureus	E.coli	S.typhi	P. aeruginosa
1.	Mannitol salt agar	Yellowish	No growth	No growth	No growth
2.	Eosin Methylene Blue	No growth	Green metallic sheen	No growth	No growth
3.	Centrimide agar	No growth	No growth	No growth	Short rod
4.	Xylose Lysine Deoxycholate	No growth	No growth	Birght pink or red appearance	No growth
5.	Macconkey agar	Pale pink to red	Dark pink in color	Pink color appear	No growh

Table 8: Antibiotics resistance pattern:

Sr. No.	Name of Antibiotics	Concentration of antibiotics (μg)	*S. aureus*	*E. coli*	*S. typhi*	*P. aeruginosa*
1.	Gentamycin	GEN^{10}	100%	100%	100%	100%
2.	Erythromycin	E^{10}	80%	100%	100%%	100%
3.	Ciprofloxacin	CIP^{5}	100%	100%	100%	100%
4.	Chloramphenicol	C^{30}	80%	100%	100%	100%
5.	Amoxyclave	AMC^{30}	80%	100%	100%	100%
6.	Ampicilin	AMP^{10}	100%	100%	100%	100%

Rupchanda Fish

Baam fish

Tillapia Fish

Catla Fish

Pankaj fish

RESULTS

Fish is the important source of protein because 60% protein found in fish. Pathogenic bacteria isolated from different fishes but some fishes contain more pathogenic bacteria to the cause disease for human being. From the present study showed that different type of microorganisms from infected fish to cause disease. This is due some antibiotic are resist to the human being.

Collection of samples:

In present study research study 60 samples were collected during period of 2019 to 2020 from fish selling Market, Akola city.

Fish names with number of samples: Rupchanda (10), Catla (*L. catla)* (10), Baamfish (*A.bengalenisis)* (10) Tillapia (*T.zilli)* (10), Pankaj (10).

Frequency distribution:
In presence study total number 60 samples were collected from fish selling market Akola. Out of 60 samples 36 samples were found to be positive. Total 61 isolates of 4 types of different bacteria were isolated.

Morphological characteristic:
The organisms were identified on the basis of morphological characterization.

Biochemical characterization:
Ability of organisms to ferment a specific carbohydrate added to basal medium results in the production of acid and gas.

Cultural characteristics:
Colonies with relevant pathogen with different morphological character and biochemical characters were selected to analyze their cultural properties and inoculated on respective media.

Antibiotic sensitivity/resistant pattern for bacterial isolate from fish part:
In vitro antibacterial susceptibility test has an important role. All the bacteria were subjected to antibiotic sensitivity and resistant test. In present antibiotic susceptibility studied for 4 types of isolates from fish part and examined toward a 6 different antibiotic by using disk diffusion method on Muller Hinton agar. The results of antibiotic susceptibility testing were categorized in two groups sensitive and resistant on the basis of zone of inhibition.

DISCUSSION

In 1994 total 271,000 ton fish production are produce in aquaculture but this fish product is damage in *Vibri anguillarum* infection in red bream, tiger puffer and olive flounder and *Vibrio spp.* Infection in various marine fishes cause serious damage every year. Disease caused by Gram positive bacteria *L.garvieae* in yellowtail (Kusuda and Kawai, 1998).

P. flurocence has been associated with septicemia and ulcerative spoilage organisms. *Pseudomonas* species is one of most serious disease in fish leading to heavy loss to fish product as well as quality of fish (Masatan, 2013).

Many general bacteria are found in marine and fresh fishes such as *S. aureus*, *Pseudomonas*, *Salmonella.* These are normal flora on different fishes but *E. coli* is opportunistic pathogen are found inside gastrointestinal tract of fish and muscles of fish (Ribeiro *et al.*, 2015).

S. aureus, E. coli and L. plantarum are common bacterial flora on fish or surrounding environment. The presence of opportunistic bacteria contaminated to the fishes due improper handling process and unhygienic condition. Some species of *lactobacillus* which are beneficial to the host but other normal flora are predominant in fishes (Echezona *et al.*, 2011).

From the study, 60 samples were collected from selling market of Akola city. In present study, four types of bacteria isolated from fish sample. Generally, aquatic zoonotic contain lot of aquatic pathogen which could be as result of their contact with different streams of their aquatic life. In this study isolates were more harmful to all fishes as well as consumers. Beta hemolysis was observed in all the bacteria. This study indicated that consumers of aquatic food products increase the possibility of zoonotic infection by handling in slaughter house which increases the contamination of fishes or fish products. In this study isolated pathogenic bacteria such as *S. typhi, S. aureus, P. aeruginosa, E. coli.* This study demonstrated that fish is potential source of infectious diseases. *E.coli* is enteropathogenic as well as opportunistic pathogen usually found in this study and other side *Salmonella* can be found in fish products as result of bacterial contamination during storage processing and consumption. And lastly *Pseudomonas* was predominantly most isolated and could be part of the transient microflora in the body of fishes.

Antibiotic susceptibility testing is used to determine the effectiveness of particular antibiotic against particular bacteria. In this study of antibiotic susceptibility testing it was observed that 4 isolated pathogens were resistant to 6 different types of antibiotics. In antibiotic resistant *E. coli* and *Salmonella typhi* was more resistant to gentamycin, and ampicillin. As compared to other organisms because some bacteria change their own environmental structure as well as intragenic structure. Isolated *S.aureus* bacteria was resist to gentamycin,

ciprofloxacin as compared to amoxicillin, erythromycin, chloramphenicol.

It has become very serious point that against Gentamycin 87% of resistance of *Staphylococcus aureus* was observed in the year 2017 by Jain and Kamble which is now becomes 100% resistance. The main reason of antibiotic resistant power increases against bacteria, reason is through mutation that occurs in DNA of cell during replication and another way that Bacteria acquire resistance through horizontal gene transfer. From the overall study it is suggested that to all consumers of fish or fish product, improvement in fish handling process is needed to minimize the prevalence of the pathogenic bacterial contamination.

Food borne disease from fish meat product causes approximately 76,000 million illnesses about 26,000 admit in hospital and 50,000 deaths from fish disease causing bacteria according to USA. Many enteropathogenic species have been found in fish such as *E. coli, S. aureus, Pseudomonas, and Salmonella.* These are food borne pathogenic bacteria produce toxin. *Salmonella* is most pathogenic bacteria to cause the typhoid fever to human. Gastrointestinal disease *V. cholerae* has worldwide distribution and found in water source *V. cholera* produce a wide spectrum of diarrhea, illness (Silva *et al.*, 2010).

Bacteria cause serious disease to the human being. The infection transmits from improper washing the meat food product. People should maintain hygienic condition before the handling process.

CONCLUSION

The determination of bacteria contaminated of fish carried out in this study was necessary in safe guarding public health. This is because fish which is contaminated with fecal material before or during harvest may cause outbreak intestinal infection disease such as typhoid fever. Since these microorganism could contaminated fish and therefore a source of food poising; harvesting, handling and cooking of fish product. Should be reduced the bacterial load.

The presence of enteric bacteria may be attributed to fecal contamination due improper disposal of sewage and water pollution. The fish acts as reservoir of human pathogen and present highly pathogenic agent such as *Salmonella*, *S. aureus, Pseudomonas* and opportunistic pathogen is potential risk to human being and may cause disease. The result obtain in this study antibiotic resistant bacteria are widespread as nearly all isolated microorganisms were resistant to most antibiotic against fish pathogen. The presence of all these microorganisms worrying finds. The bacterial associated with fish surface seen to reflect diversity of bacteria in surrounding water.

Conflicts of interest: The authors stated that no conflicts of interest.

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