

RESEARCH ARTICLE

PREVALENCE OF MULTIPLE ANTIBIOTIC RESISTANT AND HAEMOLYSIN PRODUCING BACTERIA IN MARKETED FISH SAMPLES

Subashkumar, R.*

PG and Research Department of Biotechnology,
Kongunadu Arts and Science College, Coimbatore – 641 029, Tamil Nadu, India.

ABSTRACT

Sea foods hold one of the greatest potentials to meet current and future demands of proteins to feed the world's ever-burgeoning population. Fresh seafood is an excellent source of proteins, a good source of minerals, and some vitamins, and it is low in fats, cholesterol, and sodium. Fishery products which are of great importance for human nutrition worldwide and provide clear health benefits. Food borne pathogens are the leading causes of illness and death in less developed countries killing approximately 1.8 million people annually. Bacteria are the most important cause of seafood spoilage. Percentage prevalence of bacterial population in fish samples collected from Ukkadam fish market, Coimbatore was significantly higher during the study period. About 7 fish intestinal samples were then enriched in nutrient broth and cultured. Biochemical test were performed to determine their phenotypic characteristics. The antibiotic resistance pattern and MAR index showed an increased antibiotic resistant bacterium in fish which may severe food borne illness in human. The hemolytic activity and extracellular protein reveals the frequency of virulence and strong pathogenicity. On this basis, we came to the conclusion that all the isolates are highly pathogenic and cause various food poisoning in humans.

Keywords: MAR index, Hemolytic activity, Extracellular product.

1. INTRODUCTION

Fish is one of the best supplies of proteins, vitamins and minerals and is an essential nutrient for fortifying both infant and adult diets. It is considered a healthy food and recommended by nutritionists and doctors as an ideal food to take care of almost all the nutritional requirements of man in all stages. Fish are of tremendous importance as food for people around the world, either collected from the wild or farmed in much the same way as cattle or chickens. It has high consumer preference due to its inherent nutritive values, taste and easy digestibility.

Fish is reservoir of large number of microorganisms. Bacteria are the main constituent of the gut microbiota in fish (1,2). The importance of intestinal bacteria in the nutrition and well-being of their hosts has been established for homeothermic species, such as birds and mammals (3). Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the digestive tract of fish confers a favourable culture environment for the microorganisms. Fish and fish products are eaten raw in many cultures, and these raw foods can be a route for direct transmission of pathogen. Many reports have demonstrated that Gram-negative, facultative anaerobic bacteria such as *Acinetobacter*, *Alteromonas*, *Aeromonas*, *Bacteroides*, *Cytophaga*, *Flavobacterium*, *Micrococcus*,

Moraxella, *Pseudomonas*, *Proteobacterium* and *Vibrio* spp. constitute the predominant endogenous microbiota of a variety of species of marine fish (4-9).

Foodborne illness usually arises from improper handling, preparation, or food storage. Good hygiene practices before, during, and after food preparation can reduce the chances of contracting an illness. *E.coli* was first recognized as food borne pathogen. Fish is highly perishable and should be handled with great care to preserve the natural attributes of fish and prevent microbial proliferation. With increasing demand for environment friendly aquaculture, the use of alternatives of antibiotic growth promoters in fish nutrition is now widely accepted.

2. MATERIALS AND METHODS

2.1. Collection of samples

Fish samples were collected from Ukkadam fish market, Coimbatore. Collected samples were packed in sterile plastic cover and transported to laboratory. The samples were stored in -20°C deep freezer for further use. The intestine was excised from the fish sample and was dropped into nutrient broth and overnight grown culture was taken and plated onto Nutrient agar medium and incubated at 37°C. The pure culture was grown in slants and were

*Correspondence: R. Subashkumar, Associate Professor and Head PG and Research Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore – 641 029, Tamil Nadu, India. E-mail.: rsubashkumar@gmail.com

stored in 50% glycerol and stored at -20°C deep freezer.

2.2. Biochemical test

The isolates were subjected to different biochemical tests such as staining, Indole test, methyl red test, Voges Proskauer test, citrate utilization test, carbohydrate, catalase, oxidase, motility, protease, amylase, lipase test in order to find the biochemical characteristics of microorganisms.

2.3. Antibiotic sensitivity profiling

Antibiotic sensitivity of the isolates was determined on nutrient agar medium (Himedia). About 6 different antibiotic discs were used in this study and resistance profile of the isolates was obtained. Zone of inhibition was noted. Multiple antibiotic resistances (MAR) index was also calculated.

2.4. Haemolytic activity

Blood agar medium was prepared (5 mL of blood in 100mL nutrient agar) and poured on to pre-sterilized petriplates under aseptic condition. The isolates were spot inoculated on the plates and incubated at 37°C overnight. Haemolysis was noted as zone of clearance around the isolate inoculated.

2.5. Extracellular protein precipitation

The isolates showing β haemolysis were subjected to TCA/Acetone precipitation method. About 1mL of culture supernatant was mixed with 8mL of ice cold ethanol and 1mL of Trichloro acetic acid and mixed gently. The tube was incubated at -20°C for one hour. The tube was centrifuged at 10,000rpm for 15 minutes at 4°C and the pellet

obtained was air dried and dissolved in 250 μ L of PBS.

2.6. Agar well diffusion method

Agar well diffusion was performed on blood agar plates to confirm whether the precipitated proteins had the hemolytic property. 50 μ L of the precipitated protein was added into the well and incubated at 37°C overnight. The zone of clearance was noted as hemolytic positive proteins.

3. RESULTS

3.1. Sample collection

A total of 7 fishes were collected and their biological names were identified based on their local term. Upon culturing of the fish intestine, bacterial colonies were obtained on plates and 15 isolates were selected based on the morphological characteristics of the bacteria.

Table 1. Fish samples collected for intestinal bacterial analysis

S. No.	Common name	Scientific name
1	Mullan (Toothpony)	<i>Gazzaachlamys</i>
2	Aylai (Mackerel)	<i>Rastrelligerkanagurta</i>
3	Oodalam (Flying fish)	<i>Potnichthysxingyiensis</i>
4	Kilangan (White fish)	<i>Coregonusclupeaformis</i>
5	Sangara (Red snapper)	<i>Lutjanuscampechanus</i>
6	Parai (Malabar travelly)	<i>Carangoidesmalabariws</i>
7	Ooli (Great barracuda)	<i>Sphyrneda barracuda</i>

Table 2. Biochemical characterization of the bacterial isolates

Fish	Culture name	Gram staining	Indole	Mr	Vp	Citrate utilisati on test	Carbohytrate	Oxid ase	Catal ase	Motil ity	Amyl ase	Prote ase	Lipa se
Sankara	SA 01	+	-	-	-	+	-	+	+	+	+	+	-
	SA 02	+	-	+	+	+	+	+	-	+	+	+	-
	SA 03	+	-	+	+	+	+	+	-	-	+	+	-
	SA 04	+++++	+	+	+	+	+	+	+	+	+	+	-
Kelangan	KEL 01	-	+	+	-	+	+	+	+	+	+	-	+
	KEL 02	+	-	+	-	-	+	+	-	+	+	+	-
Parai	PA 01	-	-	-	+	+	+	-	++++	+	+	+	+
	PA 02	-	+	+	-	+	+	+	-	+	+	+	+
Oodalam	OOD 01	-	+	-	+	+	+	-	+	-	+	-	+
	OOD 02	-	+	+	+	+	+	+	-	-	+	+	+
Ooli	OOL 01	-	+	+	+	-	+	+	+	-	+	-	+
	OOL 02	+	+	+	+	+	+	+	-	-	+	+	+
Iylai	IY 01	+	-	+	-	-	-	+	+	+	+	+	-
	MULL 01	-	+	+	+	+	+	+	-	+	-	+	+

3.2. Biochemical characterization

All the strains were subjected to biochemical characterization and their phenotypic characteristics were noted. Both Gram negative and Gram positive isolates were distributed among the isolates.

3.3. Antibiotic sensitivity profiling

The antibiotic resistances of isolates were checked using six different antibiotics discs and the results were tabulated. About 80% of the isolates showed resistance towards Co-Trimoxazole (25µg) and Tetracyclin (30µg), 66.7% of the isolates showed resistance to Carbenicillin (100µg), 33.3 % of isolates showed resistance to Vancomycin (30µg), 53.3% of isolates showed resistance to Cefazolin (30µg) and 60% of isolates showed resistance to Clindamycin (2µg).

Table 3. Antibigram assay for the bacterial isolates

Isolate	CO-TRI*	CAR*	TET*	VAN*	CEF*	CLI*
SA 01	+	+	+	+	+	+
SA 02	+	-	+	-	+	+
SA 03	+	+	+	-	-	-
SA 04	-	-	-	-	-	-
KEL 01	+	+	+	+	+	-
KEL 02	+	+	+	+	+	+
PA 01	+	+	+	-	+	+
PA 02	+	+	+	+	-	-
PA 03	-	-	-	-	-	-
OOD 01	+	+	+	-	+	+
OOD 02	+	+	+	-	+	-
OOL 01	+	+	+	-	-	+
OOL 02	+	+	-	-	-	+
IY 01	+	-	+	-	+	+
MUL 01	-	-	+	+	-	+

*CO-TRI – Co-Trimoxazole; CAR-Carbenicillin; TET-Tetracyclin; VAN-Vancomycin; CEF- Cefazolin; CLI-Clindamycin.

3.4. MAR index value

The MAR (Multiple Antibiotic Resistance) index was calculated and the results were tabulated. The MAR index value clearly indicates the rate of resistance and sensitivity of the isolates which is very important to find out the pathogenicity.

Table 4. MAR index value

S. No.	Bacterial isolate	MAR index value
1.	SA 01	0.83
2.	SA 02	1.2
3.	SA 03	1.0
4.	SA 04	3.0
5.	KEL 01	1.2
6.	KEL 02	1.2
7.	PA 01	0.83
8.	PA 02	2.0
9.	PA 03	0.83
10.	OOD 01	0.67
11.	OOD 02	1.2
12.	OOL 01	1.0
13.	OOL 02	1.2

14.	IY 01	1.0
15.	MUL 01	1.0

3.5. Hemolytic activity

The hemolytic activity of the isolates confirmed the pathogenicity of the organism. Out of 15 strains, 5 strains showed α - haemolysis, 4 strains showed β - haemolysis and 6 strains showed γ - haemolysis. Generally it is considered that the β - haemolytic strains are highly pathogenic.

Table 5. Haemolytic activity of the isolated strains

Culture Name	Growth	α	β	γ
SA 01	+	-	-	+
SA 02	+	-	+	-
SA 03	+	-	-	+
SA 04	+	-	-	+
KEL 01	+	+	-	-
KEL 02	+	+	-	-
PA 01	+	-	-	+
PA 02	+	+	-	-
PA 03	+	-	+	-
OOD 01	+	+	-	-
OOD 02	+	-	-	+
OOL 01	+	-	+	-
OOL 02	+	-	+	-
IY 01	+	-	-	+
MUL 01	+	+	-	-

3.6. Extracellular protein precipitation

The supernatant of the strains OOL 01, SA 02, PA 03 and OOL 02 were taken. In order to make, confirmation where the haemolytic substance produced by the isolates were proteins, the precipitated proteins were loaded on to blood agar plate which shows haemolysis positive, confirming the haemolytic substance is proteinaceous in nature.

Table 6. Confirmation of β haemolysis caused by extracellular proteins

ISOLATES	10µL	20 µL	40 µL	50 µL
OOL 01	+	+	+	+
SA 02	+	+	+	+
PA 03	+	+	+	+
OOL 02	-	+	+	+

4. DISCUSSION

Fish is considered as a healthy food and recommended by nutritionists and doctors as an ideal food to take care of almost all the nutritional requirements of man in all stages. Human infections due to many pathogenic bacteria are reported to have been transmitted through fin fish, shell fish and other sea food products. A high incidence of pathogen undoubtedly originates from the frequent consumption of Marine foods in these countries. In the present study, about 7 fish isolates collected and 15 isolates selected depend upon their phenotypical characteristics and morphology. The higher percentage of incidence were recorded which may be attributed to post-harvest contamination during handling, transportation, and selling through

fishermen and fish vendors (10,11). The antibiotic resistance and sensitivity of the isolates were checked using six different antibiotics and the isolates showed high resistance towards antibiotics. Multidrug resistance poses a serious clinical problem in the treatment of cancer and infectious diseases and is responsible for many tens of thousands of deaths each year (12,13). Increasing resistance of bacterial species to various antimicrobial agents and chlorine in potable water presents a significant threat to public health. Hemolysins are exotoxin protein produced by bacteria which causes lysis of red blood cells *in vitro*. Bacterial species is known to produce a variety of virulence factors (14,15). Among them, haemolysin is the important one, also considered as the primary toxin, produced by most of the pathogenic bacteria (11,16,17). The present study showed high haemolytic activity and strong pathogenicity. The extracellular products from hemolysin also showed virulence towards fish and human. It emphasise that the respective protein of haemolysin is able to cause the diseases in human and animals (18). Based on the results obtained and the previous reports, it is concluded that the hemolysin and antibiotic resistance profile might be used to understand the existence of virulent bacterial isolates in diverse environments. It exhibits the presence of strain diversity among the strains and species. This study indicates that the presence of *A. hydrophila* with virulence potential in fish samples collected from the study area, which may be a major threat to public health.

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