Isolation of Pathogenic and Non-pathogenic Microbial Stains from Different Types of Sea Fish Samples and their Quality Assessment with Antibiogram Properties

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AS, TA, ATK, TBE, JM and MD together planned and designed the research. Authors AS, ATK and TBE arranged the whole facilities for the research. Authors AS, TA and ATK conducted the entire laboratory works. Author AS imparted in study design and interpreted the results putting efforts on statistical analysis with authors JM, MD and TBE. All authors read and approved the final manuscript.

ABSTRACT

Aims: The contamination of sea fish and seafood by bacteria of fecal origin along with various pathogenic drug-resistant microorganisms are widely documented as the cause of several human diseases. Our present studies aim to determine the comparative microbiological quality among raw, cooked and cooked-frozen fish along with antimicrobial profiling of those isolated pathogenic and non-pathogenic microbes with potential drug modelling and increasing the awareness of taking unnecessary antibiotics. The objective of the present study was to investigate the status of fish in cold condition after cooking.

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INTRODUCTION

Aeromonas, Vibrio spp., Escherichia coli, Klebsiella spp., Salmonella spp., Shigella spp., Staphylococcus spp., Pseudomonas spp. and Vibrio spp. were noticed to be present in raw fish samples. The microbial loads were reduced in cooked samples, and the status was static in frozen samples. The study of antibiogram showed several pathogenic isolates to be drug-resistant against second-line drugs.

RESULTS: Total viable bacteria were present in all four fish samples in raw, cooked, and frozen condition up to 8.5 log CFU/ml. Most of the raw fish samples were found to harbour a vast population of microorganisms up to 7 log CFU/ml, including fecal coliforms. Several specific bacterial species like Escherichia coli, Klebsiella spp., Salmonella spp., Shigella spp., Staphylococcus spp., Pseudomonas spp. and Vibrio spp. were noticed to be present in raw samples. The microbial loads were reduced in cooked samples, and the status was static in frozen samples. The study of antibiogram showed several pathogenic isolates to be drug-resistant against second-line drugs.

CONCLUSION: Thus, the incidence of fecal coliforms in raw fish may be considered as a severe threat to public health upon consumption of such fishes. Such a prevalence of pathogens in the studied fish samples, including antibiotic-resistant strains of pathogenic microorganisms, can lead to severe public health risk and are the significant findings of this study.

Methodology: We collected a total of four sea fish species (Rupchada, Koral, Tuna, and Poma) from the local shops in Dhaka city. Raw, cooked, and frozen fish samples were analyzed for the existence of pathogenic bacteria through conventional cultural techniques and the confirmatory biochemical identification procedures. Kirby-Bauer disk diffusion method was performed to demonstrate the antibiotic susceptibility pattern of isolated microorganisms.

Keywords: Sea fish; pathogens; antibiotic resistance; microbiological quality; health risk.

1. INTRODUCTION

Animal protein, highly unsaturated fatty acid (HUFA), polyunsaturated fatty acid (PUFA), minerals, and vitamin are source of complete nutrients which play a spectacular role in human and animal growth along with other nutritional value. These types of nutritional source highly comes from fish and different types of fish products. Marine fish oil is a good source of omega-3 fatty acids [1-3]. In Bangladesh, research has shown that about 60% of the total animal protein intake is derived from fishery products [4,5]. Marine water of Bangladesh also has 442 species of fish and 36 species of marine shrimps and which is widely consumed by Bangladesh people and tourists [6]. Because of its advanced nutritive value, sea fishes constitute a significant substance for pathogenic and non-pathogenic microorganisms. Fishes can be contaminated by both aquatic environment and post-harvesting condition [7]. Due to the colonization of drug resistant pathogenic bacteria and fungi a wide ranges of sea fish’s spoilage occurs, which adversely affect the economic condition in Bangladesh, as well as public health. Contamination of sea fishes can take places at several stages of transport, handling, processing, packaging, and storage condition by both bacteria and fungi. Previous investigation has revealed that processing materials, water, and ice could be a source of contamination [8].

However, normal flora of fish contributes to spoilage due to inappropriate packaging [9-11]. Aeromonas, Vibrio spp., Pseudomonas spp., Staphylococcus spp., Salmonella spp., Listeria spp., Clostridium perfringens spp. are major cause of various foodborne illness when they enter and colonized into our intestine through contaminated sea fishes [12-15]. Along with bacteria and fungi, seafood-associated illness can be caused by viruses (Norovirus and Hepatitis A) and certain parasites. Most outbreaks of food poisoning associated with fish and seafood derive from consumption of raw or inadequately cooked food, and cross-contamination during processing [16]. Several types of pathogenic and non-pathogenic bacteria along with fungus are naturally associated with raw fishes, that can be opportunistic and cause foodborne infections rapidly if it is left for several hours at room temperature without processing. Pathogenic bacteria, especially Salmonella spp. and Vibrio spp. are the primary concern of food safety concerning seafood. When seafood is processed with uncontaminated water and cooked properly, it lowers the risk of food poisoning. Lack of proper temperature control is significant factors that can lead to pathogen growth and foodborne illness. Frozen fish are prone to contaminated by Listeria spp. [17,18]. Some thermostable enterotoxin of S. aureus and E. coli would not be destroyed completely even after cooking of seafood. Due to improper handling re-contamination can take place and consumption of this type of food causes severe food poisoning [19-24]. On the other hand, working people widely consume cooked-frozen fish in their busy life. They cook fish and keep it in the refrigerator for 3-7 days or more. During this period the shelf-life of fish, its organoleptic
and chemical properties can be changed. Presence of *Staphylococcus aureus* and *Bacillus cereus* was recorded in some cooked-frozen fish and meat as they can grow at refrigeration temperatures though at a lower growth rate [25-27]. Along this line, it was clear that microbes can be found in any of the three conditions. Also, drug resistance and virulent genes of the spoiling microflora are another important concern as they can be transferred to the pathogens colonizing the fish which may pose serious health threat especially in case of disease medication [28-31]. The extensive misuse of antibiotics led to the development of serious problems of resistance and hence limited the usefulness of antibiotics to eliminate bacterial infections [32,33]. A study showed that more than 70% of infecting pathogens were resistant against at least one of the commonly used antibiotics [34]. A habitual conductance of antibiogram thus claims its importance from the view of public health importance. Our present studies aim to determine the comparative microbiological quality among raw, cooked and cooked-frozen fish along with antimicrobial profiling of those isolated pathogenic and non-pathogenic microbes which will be useful for potential drug modelling and increasing the awareness of taking unnecessary antibiotics.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

For the analysis of microbial load, four fish samples (Rupchada, Koral, Tuna, and Poma) were collected from the local market in Dhaka city during August using a sterile aseptic container together with ice. The scientific name of Rupchada, Koral, Tuna, and Poma are *Pampus chinensis*, *Lates calcarifer*, *Thunnus albacores* and *Argyrosomus amoyensis* respectively. The experiment was conducted in triplicate and fish samples were taken from four different traders. Twenty (20) gm of raw, cooked, and cooked-frozen samples of each fish was homogenized with 180 gm of sterile normal saline. The homogenized suspension was subjected to serial dilutions (10-fold) up to $10^6$ with normal saline [35].

2.2 Enumeration of Total Viable Bacteria and Fungus

For enumerating total viable bacteria (TVB) and total fungal count 0.1 ml of each sample was spread onto Nutrient agar (NA) and Sabouraud dextrose agar (SDA). For TVB, plates were incubated at 37°C. For the fungal assay, plates were incubated at 25°C for 3 days [36].

2.3 Isolation of Total Coliform and Fecal Coliform

For isolation of coliforms, fecal 0.1 ml suspension was spread over MacConkey agar and MFC agar. For the isolation of *Escherichia coli* and *Klebsiella* spp., plates were incubated at 37°C for 18-24 hours. Presence of *E. coli* was further confirmed by the appearance of bluish-black colonies with green metallic sheen on eosin-methylene blue (EMB) agar. While for fecal coliforms, plates were incubated at 44.5°C for 24 hours [36].

2.4 Assay of Other Pathogenic Bacterial Loads

For the isolation of *Salmonella* spp., *Shigella* spp., *Vibrio* spp. and *Staphylococcus* spp., 0.1 ml of suspension was spread onto Xylose Lysine Deoxycholate (XLD) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plates and Mannitol salt agar (MSA) respectively. After incubation at 37°C for 24 h, characteristic colonies were enumerated [36].

2.5 Biochemical Analysis of Pathogenic Strains

Several biochemical tests (TSI, Indole production, MR, VP, Citrate Utilization, Catalase, Oxidase) were performed to determine and confirm the isolates by following the standard protocol for further confirmation of the presence of pathogenic bacteria [37,38].

2.6 Determination of Drug Susceptibility Pattern of Pathogenic Microorganisms

The pathogenic isolates (*Shigella* spp., *Klebsiella* spp., *Pseudomonas* spp., *Vibrio* spp. and *Staphylococcus* spp.) were examined for antibiotic susceptibility traits (either drug-resistant or sensitive) by Kirby-Bauer disc diffusion assay on Mueller-Hinton agar (Difco, Detroit, MI) against commonly used antibiotics following the standard protocol [39-42]. Based on commercial accessibility, we used different concentrated antibiotic discs such as Ampicillin (10 µg), Ciprofloxacin (5 µg), Piperacillin (25 µg), Ceftriaxone (30 µg), Amoxicillin (30 µg), Imipenem (30 µg), Chloramphenicol (30 µg),
Trimethoprim-sulfamethoxazole (25 µg), Gentamycin (10 µg), Nalidixic acid (30 µg).

2.7 Statistical Analysis

All the experiments were performed in triplicate. Statistical analyses were performed by determining the p-value through t-test.

3. RESULTS

3.1 Prevalence of Pathogenic Bacteria and Fungi in Fish Samples

The bacterial contamination was very high in raw fish samples more than the cooked and frozen fish. The total viable bacterial and fungal growth were found in raw fish (Poma fish), nearly 8.5 log CFU/ml. Total viable bacteria were also present in cooked and frozen Poma fish up to 5.5 log and 4.9 log CFU/ml respectively. In case of specific pathogens like E. coli, Klebsiella, Staphylococcus spp., Shigella spp., Salmonella spp., Pseudomonas spp. and Vibrio spp. were present within the range of 3.5-5.0 log CFU/ml. Frozen Poma fish was contamination-free in case of all pathogens, but E. coli and Pseudomonas were present in cooked Poma fish. Both raw and cooked Poma fish were found to be fecally contaminated (Fig. 1A).

In the case of Rupchanda fish, raw, cooked, and frozen samples were found to be contaminated by viable bacteria up to 6.5 log CFU/ml. Specific pathogens were noticed in raw Rupchanda fish up to 6 log CFU/ml whereas E. coli and Staphylococcus were present in frozen Rupchanda within the range of 2.5 log-3.9 log CFU/ml. The growth of Shigella was absent in all three categories of Rupchanda fish. Raw Rupchanda fish showed the existence of fecal bacteria (Fig. 1B).

Fig. 1. Microbiological status of different sea fish in a different state like Raw fish, Cooked fish and Frozen fish. Panel A is indicating the Rupchanda fish, panel B indicating the Koral fish, panel C indicating the Tuna fish and panel D indicating the Poma fish.
Table 1. Biochemical identification of the pathogenic isolates from sea fish

<table>
<thead>
<tr>
<th>Identified microorganisms</th>
<th>Slant</th>
<th>TSI</th>
<th>But</th>
<th>Gas</th>
<th>H₂S</th>
<th>M</th>
<th>Indole Production</th>
<th>MR</th>
<th>VP</th>
<th>Citrate Utilization</th>
<th>Catalase</th>
<th>Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Y</td>
<td>Y</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td><em>Klebsiella spp.</em></td>
<td>Y</td>
<td>Y</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td><em>Vibrio spp.</em></td>
<td>R</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td><em>Staphylococcus spp.</em></td>
<td>R</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>R</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>R</td>
<td>Y</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>R</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
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</table>

All the experiments have been done three times and the results were reproducible. One representative data have been shown.

TSI=Triple Sugar Iron Test, Y=Yellow (Acid), R=Red (Alkaline), MR=Methyl red, VP=Voges-Proskauve
Meanwhile, the raw Koral fish exhibited the massive array of *E. coli*, *Klebsiella*, *Staphylococcus*, *Shigella*, *Salmonella*, *Pseudomonas* and *Vibrio* to 4.5 log CFU/ml. Only *Staphylococcus* and *Salmonella* were present in cooked Koral samples up to 4.3 log CFU/ml. All three categories exhibited total viable bacteria. Only raw Koral fish exposed to fungal load (Fig. 1C).

The raw tuna fish showed massive fungal contamination as well as total viable bacteria which was recorded within the range of 5.5 to 7 log CFU/ml (Fig. 1D). In case of specific bacteria, *E. coli*, *Klebsiella*, *Staphylococcus*, *Shigella*, *Salmonella*, and *Vibrio* were present in raw tuna fish up to 4.8 log CFU/ml while *Pseudomonas* was absent in raw, cooked & frozen tuna. *Staphylococcus* spp. was present in raw, cooked, and frozen samples within the range of 3.2 log to 5.3 log CFU/ml (Fig. 1D). Only the raw tuna showed fecal contamination. However, fungal growth was observed in both categories of samples.

### 3.2 Biochemical Identification of Pathogenic Stains

In the current study, higher pathogenic loads were found in all the category of four sea fish samples employed and biochemical examination such as TSI, Indole Production, MR-VP, Citrate Utilization, Catalase, Oxidase were performed to identify the pathogenic bacteria strains. In total, seven different types of pathogenic microorganisms including *E. coli*, *Klebsiella* spp.; *Vibrio* spp.; *Staphylococcus* spp.; *Pseudomonas* spp.; *Salmonella* spp.; *Shigella* spp. were isolated and all the isolates were biochemically identified (Table 1).

#### 3.3 Antibiotic Susceptibility Patterns of Bacteria

Most of the pathogenic isolates showed higher rates of resistance against ampicillin, ciprofloxacin, amoxicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (Fig. 2A). On the other hand, the strains were found to be sensitive against imipenem, gentamycin, piperacillin, nalidixic acid, and ceftriaxone (Fig. 2B).

### 4. DISCUSSION

Spoilage of sea fish caused by different type of bacterial and fungal strain, that’s not very infrequent therefore it is very essential to ensure the quality of fish as well as the consumer's safety. Some pathogenic strains such as *Pseudomonas* spp., *Staphylococcus* spp. and *Vibrio* spp. were found in Poma, Rupchanda, Koral and Tuna fishes which were biochemically identified by performing TSI, Indole Production, MR-VP, Citrate Utilization, Catalase, Oxidase test. These pathogenic strains were also assayed for drug-resistant activity on first line and second-line antibiotics. In the previous study, researchers were able to identify a vast array of microbial growth in different sea fish samples of which most were drug-resistant [43].
remarkably reduced after cooking, and even the frozen condition. The bacterial load was significantly lower in the cooked samples compared to the raw samples. This study tried to sort out the cooking process's impact on bacterial loads.

In addition, the cooked and frozen samples were entirely satisfactory more than the raw samples. The current investigation of antibiogram revealed that the most of the pathogens were sensitive against commonly used antibiotics including ampicillin, ciprofloxacin, amoxicillin, chloramphenicol, trimethoprim-sulfamethoxazole, while sensitive against imipenem, piperacillin, nalidixic acid, gentamycin and ceftriaxone (Fig. 2) which was found quite similar as vice-versa in previously described study [43].

In additional, the cooked and frozen samples were entirely satisfactory more than the raw samples. This study tried to sort out the cooking effects on the reduction of existence microorganism in the fish samples as well as the various consequences that may happen during the frozen condition. The bacterial load was remarkably reduced after cooking, and even the quality was sustained in the frozen state. On the other hand, it's essential to evaluate the drug resistance traits of the fish pathogens to ensure consumers health through disease prevention and treatment possibility. However, the major outcome of this study is the finding that cooking at the proper temperature may reduce the microbial spoilage in food and fish. Besides the misuse of antibiotics should be prohibited to control the drug-resistant pathogens.

5. CONCLUSION
The present investigation confirmed the existence of contaminating microorganisms in sea fish, especially in raw samples. Several factors may affect the overall quality of food and fish such as contaminated ice, transportation and poor storage condition or due to cross-contamination from other fish and the misuse of antibiotics are the major reasons which drastically increase the resistance genes of pathogens against multi-drug therapy which will be alarming for future medication and treating the various bacterial and fungal infection in both human and animal community.

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![Antibiotic Sensitive Isolates](image-url)


Tango CN, Hong SS, Wang J, Oh DH. Assessment of enterotoxin production and cross-contamination of Staphylococcus aureus between food processing materials and Ready-To-Eat cooked fish paste.


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