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Microbiological study of sea fish samples collected from local markets in Dhaka city

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Abstract

Present study attempted to determine the prevalence of pathogenic microorganisms among the frozen sea fish samples: shrimp and prawn, and the canned sea fish samples: tuna and salmon, collected from local markets in Dhaka city, Bangladesh. Most of the samples were found to be heavily contaminated with pathogenic bacteria ranging from 1.6×10^5 to 6.7×10^9 cfu/g. Fungal growth was observed in all samples within a range of 1.3×10^4 - 3.8×10^6 cfu/g. The study of antibiogram showed a number of pathogenic isolates to be drug-resistant. Such a prevalence of pathogens including the antibiotic resistant ones among the studied fish samples may claim a serious public heath risk.

Introduction

Fish and fish products have long been used as a major food component for humans and animals. Fisheries sector plays an imperative role in the economic aid in Bangladesh, being the second highest source of earning foreign currencies and providing direct or indirect employment to the 10% of the total population of the country (DoF, 2005). The foremost sea fish in Bangladesh includes Tuna (*Thunnus thynnus*), Salmon (*Salmonidae oncorhynchus*), shrimp (*Metapennaeus monoceros*), prawn (*Penaeus monodon*) and some others which impart not only high nutritional values, but also have a vast popularity both to the native people and to the tourists. Sea fishes are known to be enriched by high nutritional components and concentrated source of energy (Mead *et al.*, 1986; Mol *et al.*, 2007; Dinakaran *et al.*, 2010; Kawarazuka, 2010).

Unfortunately, a huge amount of fish spoils every year in Bangladesh due to the growth and activity of pathogenic bacteria and fungi. A variety of fishes consumed regularly are prone to pathogenic spoilage especially by *Vibrio* spp., *Shigella* spp, *Salmonella* spp., streptococci, staphylococci, coliforms, *Listeria* spp., *Clostridium* spp. (Rahman *et al.*, 2012) which may get entry into the fish from their habitat or during the fish transportation and storage (Frazier and Westhoff, 1995; Eze *et al.*, 2010). A number of reports suggested that the consumption of the microbiologically spoiled seafoods might be responsible for food-borne diseases like diarrhea, salmonellosis, shigellosis, cholera and even some neurological diseases by an array of viruses, bacteria, fungi and parasites (Snowdon *et al.*, 1989; Starutch, 1991; Karunasagar *et al.*, 1994; Cray and Moon, 1995; Wallace *et al.*, 1999; WHO, 2012). Thus, with the growing importance of shrimp and prawn as the major export items from Bangladesh, it is worth to maintain the microbiological quality of these products. Therefore, it is crucial to estimate the rate of microbial spoilage and to establish the preventive strategy to ensure the general food safety.

Along these lines, present study examined the pathogenic prevalence among the frozen shrimp and prawn, and the canned tuna and salmon fish samples. The antibiotic resistance patterns of the isolated pathogens were also determined.

Materials and Methods

Study area, sampling and sample processing

Twenty shrimps and 20 prawn samples from Thatari Bazar fish market, 10 canned tuna fishes, and

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10 canned salmon fish samples from Agora Super Market were collected aseptically within January, 2012 to July, 2012. Samples were kept in sterile polyethylene bags embedded with ice and transported rapidly to the laboratory. Ten grams of each sample was transferred to 90 ml of sterile normal saline and was homogenized. The homogenized suspension was subjected to serial dilutions (10-fold) up to 10^6 with normal saline.

Assay of pathogenic load

0.1 ml of each sample was spread onto nutrient agar, membrane fecal coliform (mFC) agar, Sabouraud dextrose agar (SDA) and manitol salt agar (MSA) for enumerating total viable bacteria (TVB), total fecal coliform (TFC), fungi and *Staphylococcus aureus*, consecutively. For TVB and staphylococcal assay, plates were incubated at 37°C for 24 hours while for fecal coliforms, plates were incubated at 44.5°C for 24 hours. For fungal assay, plates were incubated at 25°C for 48 hours. For the isolation of *Escherichia coli* and *Klebsiella* spp., 0.1 ml suspension was spread over MacConkey agar and 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.

One ml of homogenized sample was transferred to 9 ml of selenite cystine broth for the enrichment of *Shigella* spp. and *Salmonella* spp., and also to the alkaline peptone water for the enrichment of *Vibrio* spp., followed by incubation at 37°C for 6 hours (Acharjee *et al*., 2013). From each of the 10^{-4} to 10^{-6} dilutions of the enriched broth, 0.1 ml of suspension was spread onto Xylose Lysine Deoxycholate (XLD) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plates. After incubation at 37°C for 24 h, characteristic colonies were enumerated. For the isolation of *Clostridium perfringens*, each sample was mixed in sterile saline in a ratio of 1:8 and was heated at 80°C for 15 min in order to kill vegetative cells. Then 1 ml of the suspension was kept in 9 ml fluid thioglycolate broth at 37°C for 4 h. Afterward, from each of the 10^{-4} to 10^{-6} dilutions, 0.1 ml of suspension was pour plated on perfringens agar medium, and incubated at 37°C in an anaerobic jar for 48 h.

To isolate *L. monocytogenes*, 0.1 ml suspension from 10^{-3}-10^{-6} dilutions were spread onto *Listeria* isolation media and incubated at 37ºC for 24 h. Colonies appeared as olive green was enumerated. Finally, a series of biochemical tests were performed following the standard method to confirm the pathogenic identification (Cappuccino and Sherman, 1996).

Determination of antimicrobial susceptibility

Isolates were tested for antibiotic susceptibility against ampicillin 10 µg, amoxicillin 10 µg, ciprofloxacin 5 µg, ceftriazone 30 µg, nalidixic acid 30 µg, imipenem 30 µg, chloramphenicol 10 µg, trimethoprime-sulfomethoxazole 25 µg, gentamycin 10 µg and piperacillin 10 µg, by the disc diffusion assay on Mueller-Hinton Agar (Difco, Detroit, MI) following the standard protocol (Bauer *et al.*, 1966; Ferraro, 2001; Munshi *et al*., 2012).

Results

Prevalence of pathogenic bacteria and fungi in fish samples

The pathogenic load was much higher in canned fish samples than those in the frozen fish samples. In case of canned fish, the total viable bacteria were found to be 3.3×10^9 cfu/g and 3.2×10^9 cfu/g for Tuna and salmon fish samples, respectively (Table 1). The bacterial load was higher in case of tuna fish detected as 4.2×10^7 cfu/g for *Pseudomonas* spp. whereas, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Listeria* spp. and *Staphylococcus* spp. were found to be 3.0×10^6 cfu/g, 2.2×10^5 cfu/g, 2.5×10^6 cfu/g, 2.6×10^5 cfu/g and 4.6×10^6 cfu/g, consecutively. For salmon, staphylococcal load was observed to be 6.7×10^9 cfu/g, while the loads of *Salmonella* spp., *Shigella* spp., *Vibrio* spp., and *Pseudomonas* spp. were 2.1×10^7 cfu/g, 3.1×10^7 cfu/g, 1.3×10^7 cfu/g and 2.6×10^7 cfu/g, consecutively.

In frozen fish samples, both the shrimp and prawn samples were free from *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp. and *Listeria* spp. The total viable bacteria were estimated to be 2.6×10^8 and 1.6×10^7 cfu/g for prawn and shrimp samples, respectively. *Vibrio* spp. and *Staphylococcus* spp. were isolated in both samples (1.3×10^6 and 3.6×10^7 cfu/g, respectively for prawn, and 1.6×10^5 and 2.6×10^5 cfu/g, respectively for shrimp samples) (Table 1). Except prawn samples, fecal coliforms were detected as 3.3×10^5 cfu/g, 1.3×10^6 cfu/g and 1.3×10^6 cfu/g for tuna, salmon and shrimp samples, consecutively. *Clostridium* spp. was not found in any of the samples. However, fungal growth was observed in both categories of samples.

Antibiotic susceptibility patterns of bacteria

Most of the pathogenic isolates showed higher rates of resistance against ampicillin, ciprofloxacin, amoxicillin, chloramphenicol and trimethoprime-sulfomethoxazole (Table 3). On the other hand, the isolates were found to be sensitive against imipenem, gentamycin, piperaciline, nalidixic acid

Table 1. Prevalence of pathogenic microorganisms in collected fish samples

Fish samples	Total viable bacteria (cfu/g)	Total fecal coliform (cfu/g)	Fungi (cfu/g)	*Pseudomonas* spp. (cfu/g)	[1]*Salmonella* spp. (cfu/g)	[1]*Shigella* spp. (cfu/g)	[1]*Vibrio* spp. (cfu/g)	*Listeria* spp. (cfu/g)	*Staphylococcus* spp. (cfu/g)	*Clostridium* spp. (cfu/g)
Canned fish										
Tuna	3.3×10^{9} (0.001)	3.3×10^{5} (0.001)	3.8×10^{6} (0.001)	4.2×10^{7} (0.001)	3.0×10^{6} (0.001)	2.2×10^{5} (0.0122)	2.5×10^{6} (0.0054)	2.6×10^{5} (0.0495)	4.6×10^{6} (0.001)	0 (0.0)
Salmon	3.2×10^{9} (0.001)	1.3×10^{6} (0.0885)	1.3×10^{4} (0.0885)	2.6×10^{7} (0.004)	2.1×10^{7} (0.0158)	3.1×10^{7} (0.001)	1.3×10^{7} (0.0885)	0 (0.0)	6.7×10^{9} (0.001)	0 (0.0)
Frozen fish										
Prawn	2.6×10^{8} (0.004)	0 (0.0)	3.6×10^{5} (0.001)	0 (0.0)	0 (0.0)	0 (0.0)	1.3×10^{6} (0.0885)	0 (0.0)	3.6×10^{7} (0.001)	0 (0.0)
Shrimp	1.6×10^{7} (0.0495)	2.3×10^{6} (0.0094)	1.6×10^{4} (0.0495)	0 (0.0)	0 (0.0)	0 (0.0)	1.6×10^{5} (0.0495)	(0.0)	2.6×10^{5} (0.004)	0 (0.0)

Average count (cfu/g) from all samples have been shown here.
[1]Bacterial load after enrichment (Prior to enrichment, the recovery was nil).
All data were statistically analyzed and were found significant ($p < 0.1$). Respective p-values have been indicated in parentheses.

Table 2. Biochemical identification of the pathogenic isolates

Assumed Pathogenic microorganisms	TSI			H_2S reaction	Indole test	MR test	VP test	Citrate test	Motility	Oxidase test
	slant	butt	gas							
Salmonella spp.	R	Y	-	+	-	+	-	-	+	
Shigella spp.	R	Y	-	-	+/-	+	-	-	-	
Vibrio spp.	Y	Y	-	-	+	+	-	+	+	+
Staphylococcus spp.	Y	R	+	+	-	+	-	+	+	-
Listeria spp.	Y	Y	-	-	-	+	+	-	+	-
Pseudomonas spp.	R	R	-	-	-	-	-	+	+	+

TSI Triple Sugar Iron Test
Y Yellow (Acid)
R Red (Alkaline)
MR Methyl red
VP Voges-Proskauer

Table 3. Antibiogram of the pathogenic isolates

Organisms	*Shigella* spp. n=20		*Salmonella* spp. n=20		*Vibrio* spp. n=30		*Listeria* spp. n=12		*Staphylococcus* spp. n=35		*Pseudomonas* spp. n=20	
Antibiotics	R	S	R	S	R	S	R	S	R	S	R	S
AMP	79%	21%	77%	23%	85%	15%	95%	5%	85.5%	14.5%	90%	10%
CIP	90%	10%	85%	15%	11%	89%	68%	32%	ND	ND	60.5%	39.5%
PIP	ND	ND	ND	ND	ND	ND	100%	0%	95%	5%	85%	15%
CEF	15%	85%	35%	65%	70%	30%	ND	ND	ND	ND	20%	80%
AMO	27%	73%	10%	90%	ND	ND	82%	18%	80%	20%	75%	25%
IPM	10%	90%	15%	85%	65%	35%	ND	ND	ND	ND	30%	70%
CHL	60%	40%	65%	35%	36%	64%	25%	75%	ND	ND	25.5%	74.5%
TMP-SUL	12%	88%	15%	85%	70%	30%	78%	22%	40%	60%	60%	40%
GEN	0%	100%	25%	75%	ND	ND	14%	86%	24.5%	75.5%	10%	90%
NALI	99%	1%	5%	95%	80%	20%	ND	ND	ND	ND	35%	65%

All the experiments have been done three times and the results were reproducible. One representative data have been shown
AMP (10 µg) = Ampicillin, AMO (10 µg) = Amoxicillin, CIP (5 µg) = Ciprofloxacin, CEF (30 µg) = Ceftriazone, NALI (30 µg) = Nalidixic acid, IPM (30 µg) = Imipenem, CHL (10 µg) = Chloramphenicol, TMP/SUL(25 µg) = Trimethoprime-sulfomethoxazole, GEN (10 µg) = Gentamycin, PIP (10 µg) = Piperaciline
ND Not done
N Number of isolates
R Resistance
S Sensitive

and ceftriazone.

Discussion

Fish and fish products are one of the favorite food items in Bangladesh. However, fish borne diseases may put the overall public health at a serious risk (Novotny *et al*., 2004; WHO, 2012). Most of the cases of morbidity and mortality have been reported due to the proliferation of bacterial pathogens (Butt *et al*., 2004). However, no detailed pathogenic study of sea fishes has been carried out in Bangladesh so far. Thus, the pathogenic study in the consumable and in the export quality fishes in the country asks for an emerging demand in the food sector for the sake of consumer safety and for the maintenance of the

overall fish quality.

The maximum bacterial counts for fresh and frozen fish samples recommended as 5×10^5 cfu/g (ICMSF, 1986). In the present study, fish samples exceed this limit for bacterial count, thereby, demonstrating a substantial risk on the public health. The quality of the fish and fish products largely depends on the interval between the harvesting and processing time. During this period, fishes continue to deteriorate (Antony *et al*., 2002). Moreover, handling and processing without maintaining asepsis result in pathogenic growth which renders the food products to be spoiled.

An important aspect revealed from the current study is that most of the pathogens were found to be resistant against commonly used antibiotics thereby demonstrating the ineffectiveness of the treatment during disease outbreaks if any. Such a situation hinders disease eradication and hence poses a fatal effect on the public health and community. However, in Bangladesh, probably due to poor settings as well as for the lack of appropriate knowledge on fish borne pathogens, the microbiological risk exposed by fish and fish products is obscure. Present study thus endeavored to establish a complete data on the pathogens associated with the fish samples studied and hence is of significance.

It is worth mentioning that, a lot of molecular studies established that the contaminated food containing pathogenic microbes may harbor virulence genes which become responsible for many of the food borne disease outbreaks (Gubala and Proll, 2006; Bhatta *et al*., 2007; Jakee *et al*., 2009; Munshi *et al*., 2012). Thus, the finding of the present study indicates the high risk of such virulent genes existence which may propagate from the habitant pathogenic isolates. Therefore, further study would unveil the molecular etiology of fish borne diseases.

Overall, the current findings reveal that shrimps, prawns, and canned tuna/salmon fish may harbor pathogenic microorganisms above the acceptable limit, indicating that these fish samples have not been protected from the microbial spoilage during handling, packaging, storage, and transport. Appropriate maintenance of microbiological quality is thus a vital aspect of quality control measures of such fishes.

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