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DIVERSITY OF BACTERIAL PATHOGENS FROM BENGALI CAMP FISH MARKET OF CHANDRAPUR, MAHARASHTRA, INDIA

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Abstract:
Present study tried to verify the occurrence of pathogenic microorganisms among the fresh water fishes (Clarius and Rohu spp.) and prawns, collected from Bengali camp fish market Chandrapur (MS), India. Most of the samples were found to be seriously contaminated with pathogenic bacteria ranging from 1.6×10^5 to 6.7×10^9 cfu/g. Fungal growth was also detected in all samples within a range of 1.3×10^4 - 3.8×10^6 propagules/g. The study of antibiogram illustrated 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 assert a severe public health menace.
Keywords: Bengali camp fish market, Pathogenic bacteria, Clarius spp., Rohu spp., Prawn, Antibiogram

Introduction:

Humans and animals have been utilizing fish as a major food component. 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). Dominant varieties of fishes such as *Clarius* sp., *Rohu* sp. and prawns (*Penaeus monodon*) are routinely catches from nearby water bodies and sold in the local market. These fishes and prawns are rich in nutritional values and have a vast popularity amongst the native people.

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. 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 foods 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). Thus, with the growing importance of fishes and prawn as the major food items, 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 locally available *Clarius* and *Rohu* fish samples and prawn samples. The antibiotic resistance patterns of the isolated pathogens were also determined.

Materials and Methods

Study area, sampling and sample processing

Ten each of *Clarius* sp., *Rohu* sp. and prawn samples were collected from Bengali camp fish market of Chandrapur city in sterilized polythene bags aseptically within January, 2014 to May, 2015 at an approximate interval of one month. 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 Membrane Fecal Coliform (MFC) nutrient 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 hours, 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 minutes 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 hours. 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 hours.

To isolate *Listerria monocytogenes*, 0.1 ml suspension from 10^{-3}–10^{-6} dilutions were spread onto Listeria isolation media and incubated at 37°C for 24 hours. 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, chloramphenicol 10 µg, and gentamycin 10 µg by the disc diffusion assay on Mueller-Hinton Agar (Difco, Detroit, MI) following the standard protocol (Bauer *et al.*, 1968; Ferraro, 2001; Munshi *et al.*, 2012).

Results

Prevalence of pathogenic bacteria and fungi in collected samples

The pathogenic load was much higher in fish samples than those in the prawn samples. In case of fishes, the total viable bacteria were found to be 3.3×10^9 cfu/g and 3.2×10^9 cfu/g for *Clarius* and *Rohu* fish samples, respectively (Table 1). The bacterial load was higher in case of *Clarius* sp. 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, respectively. For *Rohu*, 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.

Collected prawn samples were free from *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp. and *Listeria* spp. The total viable bacteria from prawn were estimated to be 2.6×10^8. *Vibrio* spp. and *Staphylococcus* spp. were isolated to be 1.3×10^6 and 3.6×10^7 cfu/g, respectively (Table 1). Except prawn samples, fecal coliforms were detected as 3.3×10^5 cfu/g and 1.3×10^6 cfu/g for *Clarius* and *Rohu* samples, consecutively. *Clostridium* spp. was not found in any of the samples.

Fungal isolates were reported from all the samples. *Clarius* sp. shows fungal load of 3.8 x 10^6 propagules/g *Rohu* sp. shows 1.3 x 10^4 propagules/g and prawn sample shows 3.6 x 10^5 propagules/g However, fungal growth was observed in all the samples.

Antibiotic susceptibility patterns of bacteria

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

Table 1. Prevalence of pathogenic microorganisms in collected fish samples

Fish samples	Total viable Bacteria (cfu/g)	Total fecal Coliform (cfu/g)	Fungi (propagules/g)	*Pseudomona* spp. (cfu/g)	*Salmonella* spp. (cfu/g)	*Shigella* spp. (cfu/g)	*Vibrio* spp. (cfu/g)	*Listeria* spp. (cfu/g)	*Staphylococcus* spp. (cfu/g)	*Clostridium* spp. (cfu/g)
Clarius spp.	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)
Rohu spp.	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)
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)

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 Antibiotics	*Shigella* spp. N=15		*Salmonella* spp. N = 15		*Vibrio* spp. N = 15		*Listeria* spp. N = 15		*Staphylococcus* spp. N = 15		*Pseudomonas* spp. N = 15	
	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%
AMO	27%	73%	10%	90%	ND	ND	82%	18%	80%	20%	75%	25%
CIP	90%	10%	85%	15%	11%	89%	68%	32%	ND	ND	60.5%	39.5%
CHL	60%	40%	65%	35%	36%	64%	25%	75%	ND	ND	25.5%	74.5%
GEN	0%	100%	25%	75%	ND	ND	14%	86%	24.5%	75.5%	10%	90%

All the experiments have been done in triplicates and the results were reproducible. One representative data have been shown.

AMP (10 µg) = Ampicillin, AMO (10 µg) = Amoxicillin, CHL (10 µg) = Chloramphenicol, GEN (10 µg) = Gentamycin, ND = Not done, N = Number of isolates, R = Resistance, S = Sensitive

Discussion

Fish is one of the favorite and easily available food items in rural India. However, fish borne diseases may put the overall public health at a serious risk (Novotny *et al.*, 2004). 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 fishes has been carried out in India so far. Thus, the pathogenic study in the consumable fishes asks for an emerging demand as priority for the sake of consumer health and maintenance of 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 India, 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.

Conclusion:

Overall, the current findings reveal that fishes and prawns may harbor pathogenic microorganisms above the acceptable limit, indicating that these fish samples have not been protected from the microbial spoilage during handling, storage, and transport. Appropriate

maintenance of microbiological quality is thus a vital aspect of quality control measures of such fishes.

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