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Studies on Isolation, Identification and Antibiogram of *Bacillus cereus* from Smoked Fish (*Phari*) in Kashmir Valley, India

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Smoked fish ('*Phari*') is highly popular to the Kashmiri and is prepared from *Cyprinus carpio* (common carp). The preparation of smoked fish appears to be unique to Kashmir. The fishes are not cleaned at all or gutted prior to smoking, which is carried out on slow burning green grass. Though, smoked fish is consumed traditionally, no literature documenting the microbial prevalence in smoked fish is available locally. The present study was aimed at first hand documentation of presence and antibiogram of *Bacillus cereus* from smoked fish (*Phari)* of Kashmir Valley. A total of 120 Smoked fish samples were collected from different market of Kashmir Valley. *Bacillus cereus* was confirmed on the basis of cultural characteristic on selective agar and different biochemical tests. Antibiotic sensitivity test was performed by disc diffusion method. Overall 51 isolates of *Bacillus cereus* was isolated from the 120 smoked fish samples screened. The gills were highly contaminated (22.5%), followed by fins (13.33%) and muscle (6.67%). AST results revealed that all the *Bacillus cereus* isolates were highly sensitive to Cephotaxime and Ciprofloxacin; moderately to Sulphamethoxazole/ Trimethoprim and highly resistant to Amoxycillin/ Clavulonic acid. Study indicated that the *Phari* available in local market should not be consumed as such, proper cooking or reheating is necessary to destroy the pathogenic microbes which are resistance to commonly used antibiotic, leading to severe microbial resistance to human.

Key words: Smoked fish, *Phari*, Foodborne, *Bacillus cereus*, Antibiogram.

Fish and fish products are one of the best source of proteins, omega-3 fatty acids, vitamins and minerals (calcium, phosphorus, iron, selenium, potassium) and are essential nutrients required for supplementing both infant and adult diets (Abdullahi *et al.* 2001). Fish constitute the traditional diet of most Asian and Pacific people and it represents about 14% of all animal protein on a global basis (Eyo, 2001; Abolagba and Melle, 2008). Fish is regarded a healthier alternative of meat due to the high content of Long Chain Polyunsaturated Fatty acids (LCPUFAs), which are associated with improving health and preventing diseases of old age. As per FAO (2006), per capita fish consumption in India was 4.8 kg/year. With the increasing cost of meat, consumers have become increasingly interested in fish as a' source of dietary protein.

However, fish products also act as a vehicle of foodborne pathogens (Harrera *et al.*

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2006). Food borne infection has recently become an emerging public health concern and millions of people suffer from food borne illness worldwide. The most of the food borne infections go undetected and unreported (Mahon, 1998). Poor processing and storage techniques, consumer lifestyle and eating habits have been attributed to the increased risk for food borne infection (Mahon, 1998). Fish, because of their soft tissues and aquatic environment are extremely susceptible to microbial contamination. There are two broad groups of public health significance bacteria (indigenous to fish and its environment, contamination by human/animal excreta or wastes) that can contaminate products of aquaculture. The level of contamination of aquaculture products by microbial flora depends on the environment and the bacteriological quality of the water, where the fish is cultured or caught, not on the fish species. Hence, the indigenous microbial populations of fish can vary significantly (Shewan, 1977). Fish muscle is an excellent substrate for bacterial growth because of its high water activity and nearly neutral pH. Bacteria are mostly present on the outer surfaces (skin, gills) and in the intestinal tract, the total counts of which varies with the temperature and environmental factors. Bacterial growth and invasion on the fish are prevented by the fish's natural defence system during life but after death the defence system breaks down and the bacteria multiply and invade the flesh. Spoilage of fish mostly occurs due to the microbial activity and also by enzymatic reactions of the fish itself. The Poikilothermic nature of fish selects for bacteria that can thrive in a wide range of temperatures. Thus, ensuring consumer safety, assurance of product and quality with regards to consumption of fish and fish products is one of the main problems of this industry today.

Fish smoking is one of the traditional fish processing methods aimed at preventing or reducing post harvest losses. Smoking is one of the oldest methods of preserving fish. Today, fish, smoking is no longer necessary but it remains very popular especially in less developed countries (Harlow, 1987). On the other hand, if the process is not handled correctly, smoked fish products can be vulnerable to the growth of pathogenic bacteria (Gonzalez Rodriguez *et at*., 2002). Smoking involves heat application to remove water and it inhibits bacterial and enzymatic actions of fish (Olokor, 2007; Abolagba and Melle, 2008; Kumolu-Johnson *et al*. 2009). Clucas and Ward, (1996) noted that apart from giving the product a desirable taste and odour, smoking provides a longer shelf-life through its anti-bacterial and oxidative effect, lowering of pH, imparting desirable colouration as well as accelerating the drying process and acting as antagonist to spoilage.

Fish constitutes an important component of the diet of people of Kashmir, India. The two major fish species found in the local water reservoirs include *Cyprinus carpio* (common carp), an exotic fish species introduced in Kashmir in 1959 (Sehgal, 1977) and *Schizothorax niger*, which is native to this region. Besides the fresh and sun-dried ('*Hu Gaard*') fish, smoked fish ('*Phari*') are highly popular to the local consumers. Kashmiri traditionally consume smoked fish as a delicacy. They have a common belief that smoked fish helps in maintaining the body warm and energetic in winter. *Phari* is prepared from *Cyprinus carpio* (common carp) which is abundantly available in all water bodies of Kashmir Valley. The process of preparation of smoked fish appears to be unique to Kashmir. The fishes are not cleaned or gutted prior to smoking, which is carried out on slow burning green grass (Mir and Dar, 2009). This way of preparation of smoked fish may introduce some additional problems with regards to storage and spoilage. Though, smoked fish is consumed traditionally, no literature documenting the microbial prevalence in smoked fish (*Phari)* is available locally. *Bacillus cereus* is ubiquitous in nature and appears to have adjusted to the environmental changes in such a way that its presence is anticipated in almost all foods, whether in the form of vegetative cells, spores or preformed toxins, thus posing a great public health threat. The diversified characteristics of *Bacillus cereus* make it unique and one of the most important food poisoning causing organisms**.** Owing to the importance of fish as an excellent and safe source of animal protein, the present study was aimed at first hand documentation of presence and antibiogram of *Bacillus cereus* from smoked fish (*Phari)* of Kashmir Valley.

MATERIALS AND METHODS

Sampling

A total of 120 Smoked fish (*Phari,* prepared from common carp) samples were collected from different market of Srinagar, Gandarbal, Bandipora. Samples were collected in sterilized sachets and transported in ice box to the Division of Veterinary Public Health Laboratory. Immediately after collection, from each sample the representative samples like fins, gills and muscles were acquired aseptically and were processed for isolation of *Bacillus cereus*.

Isolation and Identification

Samples were first inoculated in the BHI Broth and incubated at 37^{0}C for 24 hrs for enrichment. A loop full of inoculum was streaked on Mannitol Egg Yolk and polymixin B-sulphate agar plates and was incubated at 37^{o}C for 24 hours. The distinct flat pink colonies with serrated borders and surrounded by a zone of Lecithinovitellin reaction were taken as *Bacillus cereus*. The colonies were then confirmed by biochemical tests (Voges proskauer test, Catalase reaction, Indole production, Nitrate reduction, Starch Hydrolysis, fermentation of Ammonium salt sugar base viz Glucose, xylose, Arabinose, Mannitol) as described by Norris *et al.* (1981).

Antibiotic sensitivity tests

All the *Bacillus cereus* isolates were subjected to antibiotic sensitivity test by disc diffusion method. For AST fresh culture was used. Colonies were transferred into 5 ml of nutrient broth and was incubated at 37^{0}C for 18 hrs. After that a sterile cotton swab was dipped into the inoculum and the soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid. The entire surface of the Muller-Hinton agar plate was uniformly spread with the swab. Then the inoculum was allowed to dry for 10 minutes with the lid in place. The antibiotic discs were placed aseptically at least 24 mm apart from the disc kept at the centre on the Muller-Hinton agar plate. Then it was incubated at 37^{0}C for 24 hrs. The results were interpreted by measuring the zones of inhibition and the sensitivity of the isolates to different antibiotics was expressed nominally as highly sensitive, moderately sensitive and resistant based on the standard chart provided by the manufacturers. The antibiotic discs used were Amoxycillin/ Clavulonic acid (30 mcg), Gentamicin (10 mcg), Amoxycillin (30 mcg), Ampicillin (10 mcg), Cephotaxime (30 mcg), Ciprofloxacin (30 mcg), Norfloxacin (10 mcg), Enrofloxacin (10 mcg), Amikacin (10 mcg), Tetracycline (30 mcg), Erythromycin (15 mcg), Chloramphenicol (30 mcg), Sulphamethoxazole/ Trimethoprim (30 mcg) (HiMedia Laboratories Pvt. Ltd).

RESULTS AND DISCUSSION

The sample positive for *Bacillus cereus* were showed distinct flat pink colonies with serrated borders and surrounded by a zone of Lecithinovitellin reaction on MYPA agar plate. There were overall 51 isolates of *Bacillus cereus* isolated from the 120 smoked fish samples screened. The gills were highly contaminated (22.5%), followed by fins (13.33%) and muscle (6.67%). Biochemical tests revealed that the isolates were positive for Catalase reaction, citratate utilization, Nitrate reduction, Lecithovitellin reaction, glucose fermentation, Voges-proskauer test and negative for indole, Mannitol and Arabinose, which is characteristic for *Bacillus cereus*.

These observations are in agreement with that of the other researchers. Nieves *et al*. (2002) screened 554 smoked fish samples and isolated *Bacillus* spp. as second major group of contamination. Ehiziboloi *et al.* (2007) examined sixty five smoked fish samples (30 catfish and 35 *Tilapia) and recorded* 3% sample was contaminated with *Bacillus cereus*. Abolagba and Igbinevbo (2010) detected highest microbial load (450 x 10^5 cfu /g) and isolated *Bacillus* spp. from smoked fish in Nigeria. The higher levels of microorganisms identified from smoked fish purchased from the markets can be attributed to poor fish handling and improper smoking process adopted by fish mongers. Abolagba and Uwagbai (2011) isolated *Bacillus* spp. from smoke-dried fishes (*Ethmalosa fimbriata* and *Pseudotolithus elongatus*) sold in Nigeria. The present study showed that the gills were highly contaminated with *Bacillus cereus* than the external part, confirming the observation of Hasen and Olatsen (1999). Aydin *et al.* (1997) considered the presence of *Bacillus* spp. in fish resulting from contamination of water. Apun *et al.* (2006) isolated

Bacillus spp. from intestine samples of fresh fish sample. The bacterial species isolated from the intestines are also found in the water. Mahmoud *et al.* (2004) analysed skin, gills, fins and intestines of Common carp (Cyprinus carpio) and isolated *Bacillus* spp. Daboor, (2008) isolated *Bacillus* spp from pond fishes of Egypt. Dierick *et al*. (2005) reported fatal family outbreak of *Bacillus cereus* associated fish poisoning in Brazil. *Bacillus cereus* was isolated from common carp of Kashmir Valley in our laboratory (Data not published). However, information on microbial contamination of *Phari* in Kashmir valley is not available so no comparison could be possible with the findings of the present study.

The variations in microbial contamination of fish sample may be likely due to a lack of proper smoking and/ or improper hygienic and handling procedures adopted by the smoked fish processors and sellers. This is in agreement with the findings of Abolagba and Iyeru (1998) who reported that improper smoking and lack of proper hygienic handling of smoked fish products were result in a very high microbial load. The occurrence of microbes in smoked fish could be due to the fact that during storage, the fish sample reabsorbed moisture from the environment which then supported the growth of the microorganisms in addition to the contamination during handling and display on the market stalls (Christianah *et al.* 2010). All the isolates of *Bacillus cereus* in this study are important on public health point of view and hence harmful to human health if consumed.

Antibiotic sensitivity pattern of *Bacillus cereus* isolates

All the *Bacillus cereus* isolates (n=51) were highly sensitive to Cephotaxime , followed by Ciprofloxacin (94.12%), Norfloxacin (54.90%), Enrofloxacin (50.98%) and Amikacin (49.02%).

Table 1. Antibiotic Sensitivity pattern of *Bacillus cereus* isolated from Smoked fish (*Phari*) (n=51)

S. No	Name of antibiotic discs (mcg/disc)	Highly Sensitive	Moderately Sensitive	Resistant
1.	Amoxycillin/	0	9	42
2.	Clavulonic acid (30)		(17.65%)	(82.35%)
	Amoxycillin (30)	0	11	40
			(21.57%)	(78.43%)
3	Gentamicin (10)	0	13	39
			(25.49%)	(76.47%)
4.	Ampicillin (10)	0	14	37
			(27.45%)	(72.55%)
5.	Cephotaxime (30)	51	0	0
		(100%)		
6.	Ciprofloxacin (30)	48	3	0
		(94.12%)	(5.88%)	
7.	Norfloxacin (10)	28	16	7
		(54.90%)	(31.37%)	(13.73%)
8.	Enrofloxacin (10)	26	17	8
		(50.98%)	(33.33%)	(15.69%)
9.	Amikacin (10)	25	18	8
		(49.02%)	(35.29%)	(15.69%)
10.	Tetracycline (30)	14	25	10
		(27.45%)	(49.02%)	(19.61%)
11.	Erythromycin (15)	17	24	10
		(33.33%)	(47.06%)	(19.61%)
12.	Chloramphenicol	11	26	14
	(30)	(21.57%)	(50.98%)	(27.45%)
13.	Sulphamethoxazole/	7	32	12
	Trimethoprim (30)	(13.73%)	(62.75%)	(23.53%)

The isolates revealed moderate sensitivity to Sulphamethoxazole/ Trimethoprim (62.75%), followed by Chloramphenicol (50.98%), tetracycline (49.02%) and Erythromycin (47.06%).

The isolates showed the highest resistance against Amoxycillin/ Clavulonic acid (82.35%) followed by Amoxycillin (78.43%), Gentamicin (76.47%), and Ampicillin (72.55%) (Table-1.). The results supported the findings of other workers viz. Spanggard *et al.* (2003) reported antibiotic resistance in Enterobacteriaceae group of bacteria isolated from three freshwater fish farms in Denmark and found (15%) resistance against Oxytetracycline and (27%) resistance against Oxolinic acid. Kashkhedikar and Chabra (2009) reported 100% sensitivity to Ciprofloxacin, Cefuroxime, Ceftriaxone and Chloramphenicol, Gentamicin and Ufloxacin followed by Oxytetracyclin (50%) among the *A. hydrophila*. The organisms like *Enterobacteriaceae* family, *Aeromonas* spp, *Enterobacter* spp. and *Pseudomonas* spp. isolated from fish exhibit resistance to one or more antimicrobial agents. Resistance is high to Ampicillin (90.2%), Erythromycin (66.5%) and Oxytetracyclin (52.6%) but relatively low in Chloramphinecol (9.8%) and Sulpha-trimethoprim (6.4%) (Newaj *et al.* 2008). Pipova *et al.* (2009) reported that out of 90 tested *Staphylococci* isolates from fish surfaces, the highest sensitivity was shown by Gentamicin (85 strains) and Novobiocin (75 strains). However, the highest resistance was shown by Penicillin (34%) and Tetracyclin (32%). All the isolates of *Bacillus* spp. isolated from common carp (in the same laboratory, data not published) of Kashmir Valley were sensitive to ciprofloxacin but resistant to amoxycillin and erythromycin to an extent of 80% and 100%, respectively. The indiscriminate use of antibiotics and other synthetic chemotherapeutics as feed additives for fishery farm has resulted in an increased in drug resistance of microbes in fish, and a majority of these drug resistance bacteria carry transferable drug resistance factors.

Smoking increase the keeping quality of fish, however, when smoking not properly carried out, microbial growth and activities still continue, leading to the deterioration of the fish. However, considering the public health implications of the poor microbiological state of the smoked fish, particular attention should be made by the fish processors during processing, storage and handling. Study revealed that the Smoked fish (*Phari*) should not be consumed as such, proper cooking or reheating necessary to destroy the pathogenic microbes which are resistance to commonly used antibiotic, leading to severe microbial resistance to human.

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