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Bacteriological Quality of Farmed Freshwater Fish and Shellfish Meant for Export

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Bacteriological screening of 48 samples comprising of 12 samples of headless and 6 samples of whole prawn (*Macrobrachium rosenbergii*), 24 samples of processed rohu and 6 samples of whole rohu (*Labeo rohita*) obtained from fish processing factories of Kakinada and Mumbai were screened for aerobic plate count (APC) and for pathogens including *Salmonella* sp., *Vibrio cholerae, Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*. The samples of processed rohu (steak) obtained in four different batches (18 samples) were of good bacteriological quality. The APC was $\leq 10^5$ cfu/g. 16% of the samples were positive for *E. coli* (<20 cfu/g). All the samples were free from *Salmonella, V. cholerae,* coagulase positive *S. aureus* and *L. monocytogenes*. In one batch of 6 rohu samples obtained from Kakinada, three samples showed high APC ($\geq 10^5$cfu/g) and 3 samples showed high E.Coli (>20 cfu/g). However, no other pathogens were detected in any of the samples. APC was $\geq 10^5$ cfu/g in 33% of whole rohu samples obtained from Kakinada. *E. coli* (<20 cfu/g) was detected in 80% of the samples. However, all the samples were free from *Salmonella, V. cholerae, S. aureus* and *L. monocytogenes*. APC was found to be greater than 10^6 cfu/g in 33% samples of whole prawn; however, APC in headless prawn was $<10^6$ cfu/g. *Salmonella* contamination was found in 33% samples of whole and 42% samples of headless prawn when tested biochemically. However, serological testing of the presumptive positive isolates showed that whole prawn samples were free from *Salmonella* but 42% samples of headless prawn were positive and serotypes were *S. typhimurium* and *S. worthington*. *E. coli* was detected in 50% the samples of whole prawn, and 25% of the samples of headless prawn (<20 cfu/g). All the samples were free from *V. cholerae, S. aureus* and *L. monocytogenes*. The results suggested the need for implementation of good hygienic practices including HACCP and GMP for the improvement of microbial quality of aquacultured fishery products to meet international quality standards.

Keywords : Aquaculture, microbiology, *Salmonella, V. cholerae, S. aureus, L. monocytogenes*, rohu, prawn

World aquaculture output has been growing at 11% per annum and has reached 33.3 million tons in the year 1999 (Biradar *et. al*, 2001). In India also fish production by aquaculture has doubled to about 2.0 million tons during the last decade (Anon, 2001). Farmed shrimp contributes 59% by volume (65,894 metric tons) and 86% by value (US$ 806 million) of India's total shrimp exports (Anon, 2001). The state of Andhra Pradesh in India with 90,638 hectares of land under aquaculture cultivation tops in production of freshwater prawns. (Murthy, 2002). Fresh water giant prawn, *Macrobrachium rosenbergii* (scampi) is one of the major varieties that are grown in the country for which there is a huge demand. In the year 2000 – 2001, out of the total export of 7500 tonnes of scampi, 7000 tonnes was exported from Andhra Pradesh (Murthy, 2002).

Fishery products have been recognized as a major carrier of food-borne pathogens like *Salmonella* sp., *Staphylococcus aureus,*

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Vibrio cholerae, V. parahaemolyticus, Yersinia enterocolitica, Listeria monocytogenes, Campylobacter jejuni and *Escherichia coli* (Venugopal *et. al,* 1999) . The contamination can occur prior to harvest, during harvest and processing operations, distribution, storage, and during preparation of the product (Wekell *et al,* 1994). Presence of these pathogens in processed food products including frozen fishery products has been a major reason for the rejection by importing countries. These pathogens can come from various sources, such as water, feed, pond, soil, bird droppings and other live forms of surrounding ecosystems. Even the fish handlers can act as source of pathogens like *L. monocytogenes* (Jaysekaran *et al.,* 2002). In view of increasing outbreaks of foodborne diseases attributed to consumption of raw products in some countries, the microbiological safety of such seafood products needs to be ascertained.

Microbiological screening of fish samples intended for export will help in understanding the types of contaminating organisms, thereby helping to identify the critical control points for implementation of HACCP. This work will also help in validation of processes. The present study reports the microbiological quality of some processed aquacultured fishery products meant for export from India.

Materials and Methods

Frozen rohu (*Labeo rohita*) steaks ,whole rohu, whole and frozen fresh water prawn (*Macrobrachium rosenbergii*) samples brought from fish processing factories of Kakinada (Andhra Pradesh) and Mumbai were stored at -20°C. A total number of 6 whole rohu, 24 rohu steaks, 12 headless prawn and 6 whole prawn samples were collected. Each unit sample, randomly picked up from different locations of the frozen slab, weighed about 250 to 300 g.

Hi Media India, Ltd., Mumbai, supplied media used in the studies. Rabbit plasma for coagulase assay was from Becton Dickinson, France. Chemicals were from standard manufacturers.

The microbiological analysis was performed as per standard methods adopted from Bacteriological Analytical Manual Online, (2001), for detection, enumeration and identification to species level of individual organisms. Respective reference organisms were used for verification of the identification procedures employed.

Aerobic Plate Count (APC) was done by homogenising twenty five g of the sample in 225 ml of sterile physiological saline. After serial dilution up to fourth dilution, the samples were pour-plated using Plate Count Agar (PCA). The colonies were counted after 48 h of incubation of the plates at 37°C (BAM, online, 2001).

For the determination of *Salmonella,* twenty-five g of the sample was homogenized in 225 ml of lactose broth and incubated overnight at 37°C. After the initial pre-enrichment step, samples were further enriched in Selenite Cystine Broth, Tetrathionate Broth and Rappaport Vassiliadis Medium at 37°C for 24 h. A loopful of growth from each of these media was streaked on Bismuth Sulphite Agar (BSA), Xylose Lysine Desoxycholate agar (XLDA) and Hektoen Enteric agar (HEA). From BSA plate black, grey or black colonies with or without metallic sheen were selected. From XLDA plate pink colonies with or without black centre were selected, where as from HEA plate blue-green or blue colonies with or without black centre were selected for further identification. In absence of typical colonies atypical colonies were selected and used for further identification. Both atypical and typical colonies from each of these plates were further purified and identified by subsequent biochemical tests (BAM, online, 2001).

For enumerating *Vibrio cholerae* twenty-five g of the sample were homogenized in 225 ml of Alkaline Peptone Water (APW) and

incubated overnight at 37°C. After secondary enrichment for 6 h in APW, a loopful of the culture from primary and secondary culture media was streaked on Thiosulphate Citrate Bile Sucrose Agar (TCBS). Yellow, translucent colonies from each plate were picked up for further biochemical tests.

Enumeration of *E. coli* was performed using a 5-tube Most Probable Number (MPN) technique that consisted of pre-enrichment in Lauryl Sulphate Tryptose Broth (LSTB), selective enrichment at 45.5°C in *Escherichia coli* Broth (ECB), selective plating on Eosin Methylene Blue (EMB) agar. Purple coloured colonies with metallic sheen were selected and presence of *E.coli* was confirmed by Indole, Methyl Red, Voges-Proskauer and Citrate (IMViC) tests. The final enumeration of *E.coli* was done following McCardy's table.

Staphylococcus aureus determination was made as follows. Dilutions made for the Aerobic Plate Count were plated on Baird Parker's agar (BPA) After 24-48 h of incubation at 37°C, the characteristic black colonies with a peripheral clearance zone were counted and typical isolates were tested for coagulase activity. Coagulase activity was checked by Tube Coagulase Test as per BAM, online (2001) protocol.

For *Listeria monocytogenes* twenty-five g of the sample was homogenized in 225 ml of Buffer Peptone Water and incubated at 30°C for 24 h, followed by secondary enrichment in *Listeria* enrichment broth medium (LEBM) at 30°C for 2 and 7 days. Loopful of culture was streaked on to Listeria Selective Agar (LSA) plates. Plates were incubated at 37°C for 48 h. Pinpoint, greenish, translucent colonies were picked up for further identification. These colonies were gram stained and colonies showing gram-positive, very thin, short rods with rounded ends were selected for further biochemical tests (BAM, Online, 2001).

Results and Discussion

The 12 samples of processed rohu (steak) obtained from EU approved Mumbai plant were of very good bacteriological quality. All the samples were free from *E.Coli Salmonella, V. cholerae, S. aureus* and *L. monocytogenes* and APC was also $<10^5$ cfu/g. This indicates that implementation of GMP, GHP and HACCP ensures the safety of the product. Out of two batches obtained from Kakinada, bacteriological quality of the samples from one batch was good. All the samples showed APC$<10^5$ cfu/g. Although 3 samples were positive for *E.Coli*, the count was less than 20 cfu/g. However, the other batch comprising of 6 samples was of poor bacteriological quality, 3 out of 6 samples showed high APC ($\geq 10^5$ cfu/g) and 4 out of 6 samples showed high *E.Coli* (>20 cfu/g). The whole rohu samples obtained from Kakinada exhibited poor bacteriological quality. APC was $\geq 5 \times 10^5$ cfu/g in 33% samples and 80 % of the samples were contaminated with *E. coli* (<20 cfu/g) (Table 1). However, all the samples were free from *Salmonella, V. cholerae, S. aureus* and *L. monocytogenes*. The whole samples of rohu showed higher microbial load probably due to contamination from the intestine during analysis. It is known that gut region have

Table 1. Bacteriological profile of ready to export rohu

Bacteriological parameters	Rohu	
	Whole (cfu/g)	Steak (cfu/g)
APC	$10^3 - 10^6$ [a]	$10^3 - 10^5$ [b]
Escherichia coli	+Ve [c]	+Ve[d]
S. aureus (Coagulase positive)	Nil	Nil
Salmonella	Nil	Nil
Vibrio cholerae	Nil	Nil
Vibrio parahaemolyticus	Nil	Nil
Listeria monocytogenes	Nil	Nil
Yersinia enterocolitica	Nil	Nil

a: 3 out of 6 samples were exceeding $5x10^5$
b; 3 out of 24 samples exceeding $5x10^5$
c: 2samples < 20cfu/g 3 samples > 20 cfu/g out of 6 samples
d; 4 samples < 20 cfu/g, 5 samples > 20 cfu/g out of 24 samples

high bacterial load, whereas, in the processed rohu bacterial load is less because the fish is washed thoroughly after gutting and before processing.

Bacteriological profile of whole and headless freshwater prawns is shown in Table 2. In 33% samples of whole prawn APC was $\geq 10^6$ cfu/g. *Salmonella* contamination was observed in 33% samples of whole and 42% samples of headless prawn when tested biochemically. However, serological testing of the presumptive positive isolates showed that whole prawn samples were free of *Salmonella* but 42% samples of headless prawn were positive and serotypes were *S. typhimurium* and *S. Worthington.* Out of 2 batches of headless prawns obtained 5 samples out of 6 from only one batch was positive for *Salmonella*. This level of contamination is very high and it could be due to lack of GMP or GHP on the farm or it could be due to unhygienic conditions in the processing plant. *E.coli* was present in all the samples of whole prawn; however, the counts were <20 cfu/g. 25% of the samples of headless prawns were also positive for *E.coli* but the counts were <20 cfu/g. All the samples were free from *V. cholerae, S.aureus* and *L. monocytogenes*. The poor bacteriological quality of the prawns may be due to the feeding habit of the crustaceans that results in the concentration of pathogens.

Earlier studies on the bacteriological quality of rohu marketed in India have shown high level of bacteriological contamination with 33 to 40% samples positive for *E.Coli.* However, no salmonella contamination was reported (Sinha *et al.*, 1991; Sapna *et al*, 2001). None of the 48 samples tested in this study were contaminated with *L. monocytogenes*. Earlier study from our laboratory also could not detect *L monocytogenes* from any seafood and only *L. innocua* and *L. grayi* were present (Kamat & Nair, 1994). Recently it was reported that 44.4% of the headless marine shrimp samples were found to be contaminated with Listeria spp. (Jayasekaran *et al.*, 2002a).

Export Inspection Council of India (EIC) has stipulated bacteriological specifications for frozen marine shrimps (Table 3). However, no similar specifications are available for the fresh water aquaculture products. The present results suggest that processed rohu samples are of better microbiological quality than the whole fish probably due to the GMP and SSOP followed in the plant during processing. The comparatively poor quality of freshwater prawn reflects the poor quality of the environment, unhygienic handling and the cultivating conditions such as feed quality. It has been

Table 2. Bacteriological profile of ready to export Whole & Head less fresh water prawn

Bacteriological parameters	Aquaculture shrimps Whole (cfu/g)	Head less (cfu/g)
Aerobic Plate Count	10^4-10^6 cfu/g [a]	$1.0X10^4$–$5.0X10^5$ cfu/g [b]
Staphylococcus aureus (Coagulase positive)	Nil	Nil
Escherichia coli	+ve[c]	+ve [d]
Salmonella	+[e]	+[f]
Vibrio cholerae	Not detected	Not detected
Vibrio parahaemolyticus	Not detected	Not detected
Listeria monocytogenes	Not detected	Not detected
Yersinia enterocolitica	Not detected	Not detected

a: 3 out of 6 samples were exceeding $5x10^5$
b: out of 12 samples none were exceeding $5x10^5$
c: 3 out of 6 samples were < 20 cfu/g
d: 3 out of 12 samples < 20 cfu/g
e: 2 out of 6 samples positive
f: 5 out of 12 samples positive

Table 3. Bacteriological specification by Export Inspection Council, India, for frozen shrimp (Export Inspection council of India, Order and Rules (1995))

Aerobic Plate Count at 37°C	5 x 10^5 cfu per g ($2x10^5$ per g for products meant for raw consumption)
Escherichia coli	20 cfu per g
Staphylococcus aureus (Coagulase positive)	100 cfu per g
Salmonella	Nil in 25 g
Vibrio cholerae	Nil in 25 g

recognized that use of wastewater for fish farming or the practice of fertilizing ponds with animal manure may result in transmission of pathogenic bacteria and parasites to fish. Aquatic birds are known to harbour pathogenic strains of *V. cholerae and Salmonella* sp. that can infect the fish farms (Ogg, 1989, Beveridge, 1989). The poor hygienic quality of fishmeal used for aquafeed production may be a source of contamination of these pathogens. Our results indicate that while farming prawns better care needs to be taken to avoid contamination of the environment. The present results suggest that whole rohu samples and prawn were of poor bacteriological quality and there is a batch-to-batch variation in microbial quality. GMP (Good Manufacturing Practices) and SSOP (Sanitation Standard Operating Procedures) followed in the plant during processing of the fish may be inconsistent. The presence of *Salmonella* and coliforms in freshwater prawn reflects the poor quality of the environment, unhygienic handling and the feeding habits of the prawns. The study indicates that during fresh water aquaculture of prawns better care needs to be taken to avoid contamination from environment.

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