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Antibiotic Susceptibility of Staphylococci Isolated from *Labeo rohita* sold in Burla Fish Market, Orissa

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Abstract

Screening of commercial sold freshwater *Labeo rohita* revealed that the staphylococcal count decreased from one to two log cycles/g upon washing the fish. The fish samples harboured more number of staphylococci in edible meat portion than in guts. The microscopic, biochemical and oxidative fermentations tests of the isolates by conventional and rapid detection methods revealed that they are *Staphylococcus haemolyticus*, *S. auricularius* and *S. caseolyticus*. When the isolates were compared with 45 different antibiotics the results varied from susceptible to resistant in comparison with American Type Culture Collection cultures. In comparison to Clinical Laboratory Standards Institute isolates both the staphylococcal test cultures were resistant to penicillin (G) and vancomycin. With other antibiotics *viz*., Pristinamycin, Ticarcillin/Clavulanic acid, Gatifloxacin, Clindamycin, Clarithromycin, Levofloxacin, Linezolid, Cefeprime, Erythromycin, Streptomycin, Fosfomycin, and Piperacillin/Tazobactam the results varied from intermediate to sensitive.

Introduction

Fish form a rich source of animal protein available at an affordable price to all sections of the society and provide a means to tide over the nutritional difficulties of man. Importance of fish as a source of high quality, balanced, and easily digestible protein is well understood (Nair and Mathew 2000). In the last six decades fish production systems in the inland waters have expanded, diversified, intensified, and technologically advanced. According to Sugunan's (2002) estimate one million tons of fish are available in the inland open water systems such as rivers, estuaries, lagoons, flood plains, wetlands, and reservoirs (Sugunan 2002). Fisheries play a very important role in the country's economy in generating much needed foreign exchange (nearly 72,000 million rupees), providing employment to millions of people and also in enhancing nutritional status of the people, especially, those who are residing in hinterlands (Sahu 2004).

The reports on quality analyses pertained to freshwater fish of *Labeo* spp., namely

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Labeo rohita (Shasini 2004) and *Labeo calbasu* (Sahu 2004) cautioned on the need for studies in view of occurrence of staphylococci in commercial fish samples of wet fish markets.

Staphylococci are inherently susceptible to most antibiotics except those with purely anti-Gram-negative spectra. However, staphylococci remain frequent causes of morbidity and mortality, having proved extremely adept at developing resistance, both by mutation and by DNA transfer. *Staphylococcus aureus* is a classical pathogen, causing infections at many sites (Waldvogel 1995; Lowry, 1998). Studies on antibiotic resistance of different species of Staphylococci were carried, but are confined to isolates obtained from cattle, cats, dogs, ducks, guinea pigs, horses, mink, pigeons, pigs, rabbits, and turkeys (Schwarz et al. 1998). Reports on antibiotic resistance of Staphylococci isolated from freshwater fish are scanty. Methicillin-resistant *Staphylococcus aureus* (MRSA) is well recognized as a major cause of infection in the healthcare setting but as a matter of great concern is now emerging in the community. The glycopeptides notably, vancomycin have traditionally been the mainstay of treatment of MRSA but overuse has led to the emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) (Appelbaum 2006).

In the light of the above, studies were undertaken on screening of freshwater *Labeo rohita* sold in wet fish market of Burla, Orissa, for staphylococci. The Fish samples were screened for staphylococci before and after cleaning of the same fish and also gut to see the difference between edible meat portion and gut of the fish. Besides carrying out quantitative estimation of staphylococci, the isolates of staphylococcal groups were characterized and were compared with 45 antibiotics.

Materials and methods

Collection of sample

Fish samples were collected from the local outlet of Burla in fresh condition in sterile polythene bag (200 gauge) for immediate analysis in the laboratory. The fish samples *Labeo rohita* collected from market of one source constituted one sample.

Screening of fresh Labeo rohita for staphylococci

Each sample weighed 20g fish and from this sampling was done for estimation of staphylococci. In screening the fish before and after washing, in edible meat portion and in gut for staphylococci, Baird Parker Agar was employed (ICMSF, 1978).

Characterization of the suspected staphylococcal isolate by rapid detection methods

The organisms to be identified were isolated by standard procedure on a common medium like Nutrient Agar or Soyabean Casein digest Agar. A single isolated colony was inoculated in brain Heart Infusion broth. A biochemical test kit for identification and differentiation of genus staphylococcus was employed in the present study. The kit uses standardized, colorimetric identification system utilizing twelve conventional biochemical tests *viz*., Voges-proskaeur's Esculin hydrolysis, PYR, ONPG, Arginine utilization, Glucose, Ribose, Arabinose, Sucrose, Sorbitol, Mannitol and Raffinose, which are based on the principle of pH change and substrate utilization. Each well was inoculated with 50 µL of the above test culture inoculum by surface inoculation method. On incubation, the metabolic changes of the test cultures resulted in change in colour in the media that was either visible spontaneously or after addition of a reagent. The cultures were also examined further for citrate utilization, arginine dihydrolase test, oxidase test, catalase test, haemolysis test, growth at high pH, and differentiation test of staphylococcus strain (Schleifer 1986).

Study of antibiotic sensitivity of staphylococcus strains isolated from Labeo rohita

The agar diffusion method using Mueller Hinton was followed for challenging test cultures against different antibiotics in different concentrations.

Results and Discussion

The original source of the fish samples is Hirakud Reservoir. After the harvest, the fishes are packed in bamboo baskets and are transported to the market in iced condition for further sale.

Staphylococcal examination of fresh Labeo rohita

The results of staphylococcal examination of edible meat portions of fish (skin and flesh) before and after washing and gut are shown in Table 1. The Staphylococcal count decreased from one to two logs on washing the fish. The fish samples harboured more number of staphylococci in edible meat portion than in guts. Fish is harvested from relatively cleaner environments, however, during post harvest handling, bacteria of spoilage type, hygiene indicator and human health hazard type come in contact with the fish (Prasad and Rao, 1993). This study has shown that simple washing of fish will reduce the bacterial load by one to two log cycles. The studies of Sanjeev and Surendran (1996) revealed that fish and fishery products are good sources for staphylococcal food poisoning. According to Bergdoll (1979) the acceptable level of staphylococcal counts in fish product is six logs and above, however, in the fish samples under study the counts of staphylococci were below the dangerous level to cause any human health hazard.

Table 1. Occurrence of staphylococci in commercially sold *Labeo rohita*

No of samples	Occurrence of staphylococci /g of the sample		
	Before washing	After washing	in gut
1	8.5×10^4	5.0×10^3	$<10^1$
2	4.0×10^3	3.0×10^3	6.0×10^3
3	7.8×10^4	OG	4.4×10^3
4	9.3×10^4	3.4×10^3	6.1×10^3
5	2.7×10^5	OG	1.0×10^3

Staphylococcal counts are averages of triplicate determinations. OG: Over growth

Characterization and identification of isolates

Gram's staining of the test culture confirmed that the isolates were Gram-positive cocci. The Voges-proskauer's esculin hydrolysis, PYR, ONPG, arginine utilization, oxidase, catalase, citrate utilization, lysine decarboxylase, haemolysis and growth at pH 9.6 varied between the isolates. The test culture was subjected to aerobic and anaerobic utilization of 21 different carbohydrates. In the present study, the test cultures utilized sucrose, maltose, D-trehlose, β-D fructose and with other carbohydrates such as lactose, raffinose, dextrose, maltose, salicin, sorbitol, lactose in both aerobic and anaerobic conditions the results varied between the isolates. The morphological, biochemical, and carbohydrate utilization tests of isolates from fish samples resemble *Staphylococcus haemolyticus*, *S. auricularius*, and *S. caseolyticus* (Schleifer 1986). Coagulase-negative staphylococci (CNS) include *Staphylococcus epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. auricularius*, *S. caseolyticus* and a number of other species. CNS are important as causes of line-associated infections in the immunosuppressed and account for many of the bacteraemic episodes in neutropenic patients (Hamory et al, 1987; Oppenheim 1998). Overall, CNS account for approximately 7 to 9% of bacteraemias reported to the Public Health Laboratory Service (Reacher et al. 2000) and are important also as causes of prosthetic valve endocarditis, being more frequent than *S. aureus* in this setting.

Antibiotic sensitivity of test culture

Staphylococci are ubiquitous in nature and *Staphylococus aureus* is the most pathogenic species. The spread of antibiotic resistance among *S. aureus* strains is a major concern in the treatment of staphylococcal infections (Ito et al. 2003). The spread of MRSA from the hospital to the community setting, coupled with the emergence of VISA and VRSA, has become a major cause of concern among clinicians and microbiologists (Appelbaum 2006).

Comparison of Staphylococcus isolates of the present study Staph I and Staph II to American Type Culture Collection cultures. In comparison to Clinical Laboratory Standards Institute (ATCC)

The response of test cultures when challenged with different antibiotics and the comparison with *S. aureus* of ATCC is shown in Table 2. For convenience of interpretation the range of antibiotic response ATCC is divided into mainly three categories. The interpretation zone between the lower and upper ranges was drawn and below the mean expressed as <, above the mean as >, less than equal to mean as ≤ and more than equal to mean ≥. Accordingly the Staph I test isolate was < to 80%, 15.6% towards ≥ and ≤ 4.4% to the antibiotics tested in comparison to the ATCC. Similarly Staph II test culture is < 77.77% and 33.33% towards ≤ to different antibiotics tested. Schwarz et al (1998) observed that majority of the staphylococcal isolates that have shown resistance to tetracycline was also resistant to one or more antibiotics. The isolates of the present study showed variation in comparison with ATCC cultures (Table 2) where one is resistant another one is sensitive to tetracycline. However, both were susceptible to chlortetracycline (Table 2). Reports indicate resistance of staphylococci to teicoplanin (Livemore, 2000). In the present study too the staphylococcal isolates were resistant to teicoplanin (Table 2).

Table 2. Antibiotics tested, code, level of concentration and the zones of interpretation

Sl No.	Antibiotic employed	Code	Conc (µ)	ATCC25923*
1	Nitrofurantoin	Nf	300	18-22(<,<)
2	Fusidic acid	Fc	30	26-37(<,<)
3	Sparfloxacin	Sc	5	27-33(<,<)
4	Pristinamycin	Pm	15	23-29(<,<)
5	Penicillin (G)	P	10 IU	26-37(≥,≥)
6	Moxifloxacin	Mo	5	28-35(<,<)
7	Sulphafurazole	Sf	300	24-34(<,<)
8	Furazolidone	Fr	50	18-22(<,<)
9	Amoxycillin	Am	10	28-36(<,≥)
10	Sulphaphenazole	Sp	200	24-34(<,<)
11	Tricarcillin/Clavulanic acid	Tc	75/10	29-37(≥,<)
12	Furaxone	Fx	100	18-22(<,<)
13	Chloramphenical	C	30	19-26(≥,≥)
14	Gatifloxacin	Gf	10	27-33(<,<)
15	Clindamycin	Cd	2	24-30(<,<)

16	Cefaclor	Cj	30	27-31(**<**,<)
17	Clarithromycin	Cw	15	26-32(**<**,<)
18	Ceftriaxone	Ci	10	22-28(**<**,<)
19	Levofloxacin	Le	5	25-30(**<**,<)
20	Cephotaxime	Ce	30	25-31(**<**,<)
21	Tetracycline	T	30	24-30(**≤**,≥)
22	Linezolid	Lz	30	27-31(**<**,<)
23	Cefeprime	Cpm	30	23-29(**<**,<)
24	Erythromycin	E	15	22-30(**<**,≥)
25	Vancomycin	V	30	17-21(**<**,<)
26	Pipemedic acid	Pa	30	13-19(**≥**,≥)
27	Sulphamethizole	Sm	300	24-34(**<**,<)
28	Amikacin	Ak	10	18-24(**<**,<)
29	Teicoplanin	Te	30	15-21(**<**,<)
30	Trimethoprim	Tr	30	19-26(**<**,<)
31	Ciprofloxacin	Cf	10	27-35(**<**,<)
32	Netillin	Nt	10	22-31(**<**,<)
33	Tobramycin	Tb	10	19-27(**<**,<)
34	Gentamycin	G	50	25-33(**<**,<)
35	Streptomycin	S	10	14-22(**<**,≥)
36	Norfloxacin	Nx	10	17-28(**≥**,≥)
37	Methanamine mandalate	Me	3	14-22(**<**,≥)
38	Ampicillin (Cloxacillin)	Ax	10	35-37(**<**,<)
39	Cephatoxime	Ce	10	25-31(**<**,<)
40	Floxidin	Fl	20	25-30(**<**,<)
41	Fosfomycin	Fo	50	25-33(**<**,≥)
42	Framycetin	F	100	18-24(**<**,<)
43	Chlortetracycline	Ct	30	19-28(**≥**,≥)
44	Pefloxacin	Pf	5	24-28(**<**,<)
45	Piperacillin/Tazobactam	Pt	100/10	27-36(**<**,<)

**Staphylococcus aureus* Parentheses: Staph1 in bold & Staph2 in normal. The interpretation zone between the lower and upper ranges was drawn and below the mean expressed as <, above the mean as >, less than equal to mean as ≤ and more than equal to mean ≥.

Comparison of Staphylococcus isolates of the present study Staph I and Staph II to CLSI (Clinical Laboratory Standards Institute) standards

In comparison with CLSI standards both the test isolates were sensitive to Ticarcillin/Clavulanic acid, Clarithromycin, Levofloxacin, Linezolid, Fosfomycin,

Piperacillin/Tazobactam, intermediate to Clindamycin, Cefeprime and resistant to penicillin (G) and Vancomycin (Table 3). With other antibiotics the response varied between the cultures.

Table 3. Results of antibiotics sensitivity of Staph I and II isolates of the present study in comparison to CLSI (Clinical Laboratory Standards Institute) tested *Staphylococcus aureus* type cultures

Sl No	Antibiotic employed	Code	Conc(µ)	Zone of interpretation in mm			Test results	
				Sensitive	Intermediate	Resistant	Staph1	Staph 2
1	Pristinamycin	Pm	15	19	16-18	15	Intermediate	Sensitive
2	Penicillin (G)	P	10 IU	29	20-27*	28	Resistant	Sensitive
3	Moxifloxacin	Mo	5	24	21-23	20	Intermediate	Resistant
4	Ticarcillin/Clavulanic acid	Tc	75/10	24-30*	23	14*	Sensitive	Sensitive
5	Gatifloxacin	Gf	10	23	20-22	19	Sensitive	Resistant
6	Clindamycin	Cd	2	21	15-20	14	Intermediate	Intermediate
7	Clarithromycin	Cw	15	18	14-17	13	Sensitive	Sensitive
8	Levofloxacin	Le	5	19	16-18	15	Sensitive	Sensitive
9	Linezolid	Lz	30	21	NA	NA	Sensitive	Sensitive
10	Cefeprime	Cpm	30	18	15-17	14	Intermediate	Intermediate
11	Erythromycin	E	15	23	14-22	13	Intermediate	Sensitive
12	Vancomycin	V	30	15	NA	NA	Resistant	Resistant
13	Streptomycin	S	10	15*	12-14*	11*	Intermediate	Sensitive
14	Fosfomycin	Fo	50	16*	13-15*	12*	Sensitive	Sensitive
15	Piperacillin/Tazobactam	Pt	100/10	18	18-20*	17	Sensitive	Sensitive

*Standards not pertain to *Staphylococcus aureus* type cultures. NA: Not available. In case of Vancomycin no zone is seen (total resistance)

Both the test cultures are resistant towards vancomycin. The first clinical isolate of VISA was identified in 1997, and these strains have now been reported worldwide (Hiramatsu et al. 1997). Slackening in hygiene can lead to drug resistance in *S. aureus* (Livermore 2000) and fish and fish curing environs all the more the source of drug resistant staphylococci owing to the unhygienic conditions. One of the important ways to tackle this problem is continuous monitoring of fish and fish curing environments for antibiotic resistant staphylococci. More recently, there have been reports of VRSA, which is even more alarming, as these isolates demonstrate complete vancomycin resistance (Kacica and McDonald, 2004). Antibiotic susceptibility studies in *Staphylococcus aureus* are gaining importance due to emergence of vancomycin resistance strains all over the world. The mechanisms underlying vancomycin resistance are not yet fully understood, changes to the bacterial cell wall the site of action of the

glycopeptides are believed to be key. Recent evidence also supports the transfer of genetic material among bacteria as contributing to the development of VRSA. Based on the cases identified to date, risk factors for the development of VRSA may include old age, compromised blood flow to the lower limbs, and the presence of chronic ulcers (Appelbaum 2006). In the absence of surveillance programs and possible limitations of automated and non-automated detection methods, many cases of VISA and VRSA infection go undetected. In this regard it shall be noted that immunocompromised and under nourished fisher folk, especially pregnant, lactating women, and old aged groups are more vulnerable to these kinds of infections. Hence, knowledge of antibiotic susceptibility pattern of the isolates is important for future studies.

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