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Biotyping and Antibiotic Resistance Profile of *Yersinia enterocolitica* Associated with Seafoods from South-west Coast of India

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Abstract

This study reports occurrence of *Yersinia enterocolitica* biotype 1A strains resistant to ampicillin, cephalothin and sulphamethizole in fish and shellfish species landed in southwest coast of India. Thirty finfish and shellfish samples collected from various sources such as farms, retail outlets and landing centres in south-west coast of India were examined for the presence of *Y. enterocolitica*. Sixty three *Yersinia* strains were isolated and characterized. *Y. intermedia* was the most commonly isolated species. *Y. enterocolitica* biotype 1A was recovered from squid, but did not belong to the pathogenic serogroups O:3, O:5, O:8 or O:9. The antibiotic susceptibility tests of the isolated *Y. enterocolitica* strains revealed that these strains were resistant (100%) to ampicillin, cephalothin and sulphamethizole. As determined through PCR analysis the *Y. enterocolitica* isolates from squid carried *inv* genes, but were negative for *ail* and *yst* genes.

Key words: *Yersinia enterocolitica* biotype 1A, antibiotic resistance, shellfish

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Introduction

Yersinia enterocolitica, a psychrotrophic pathogen, is recognized world wide as an important emerging human pathogen. Currently, *Y. enterocolitica* is represented by six biotypes 1A, 1B and 2–5, of which biotypes 1B and 2-5 are regarded as primary pathogens (Bottone et al., 2005). However, biotype 1A strains of *Y. enterocolitica* which lack virulent markers such as chromosomal invasion-associated genes and plasmid have been implicated as a cause of gastrointestinal disease and have been isolated from patients with diarrhea in Canada, Australia, New Zealand, Switzerland, South Africa, Republic of Georgia and USA (Butt et al., 1991; Burnens et al., 1996; Grant et al., 1998). A nosocomial outbreak of gastroenteritis in Canada involving nine patients was attributed to a strain of *Y. enterocolitica* biotype 1A belonging to serogroup O:5 (Ratnam et al., 1982). *Y. enterocolitica* is commonly transmitted to humans by contaminated food and water. The prevalence of *Y. enterocolitica* in fish and shellfish from temperate areas is well documented (Morris & Feeley, 1976; Peixotto et al., 1979; Chiesa et al., 1987; Velázquez et al., 1996). The ability to survive and multiply in foods at refrigeration temperatures add to the significance of *Y. enterocolitica* in seafoods.

In India, a food-borne outbreak of yersiniosis by biotype 1A strains of *Y. enterocolitica* was reported in North Arcot district of Tamil Nadu involving many persons (Abraham et al., 1997) and the vehicle in this outbreak was identified as contaminated well water used to dilute buttermilk. *Y. enterocolitica* biotype 1A was isolated from diarrhoetic stools of paediatric patients and various aquatic sources such as river, wastewater and ground water in Delhi, India (Singh et al., 2003). Because of the wide spread occurrence and persistence of *Y. enterocolitica* in aquatic environment such as well water from Tamil Nadu, waste water from New Delhi and river water of Yamuna, this pathogen is of potential importance in India. Swine is the primary reservoir of

Y. enterocolitica. Aquaculture integrated with animal husbandry is practiced in many Asian countries including India. Hence, the study was undertaken to investigate the prevalence of *Y. enterocolitica* in finfish and shellfish from retail outlets, landing centres and farms, identification of prevalent biogroups as well as determination of their pathogenicity potential and antibiotic resistance profile. The increase in antimicrobial resistance in pathogenic bacteria due to exchange of genetic material in the natural environment is an issue of concern (Davison, 1999). The results of the present study provide a baseline data for *Y. enterocolitica* biotype 1A and antimicrobial resistance profile for management of resistance.

Materials and Methods

Samples

Eighteen fish (marine, brackishwater and freshwater fish) and twelve shellfish comprising of crustaceans (shrimp and crab), bivalves (black clam) and cephalopods (squid, cuttlefish, and octopus) collected from various sources such as aquaculture farms, retail outlets and landing centres in south west coast of India were studied. The samples were transported to the laboratory under aseptic conditions in sterile polythene pouches kept on ice where they were immediately analyzed.

Isolation and identification

Samples of muscle tissue (10 g) were aseptically weighed into 90 ml Peptone Sorbitol Bile Broth (PSBB; pH 7.6), homogenized for 30s with a stomacher (Lab blender 400, Seward Ltd., UK) and incubated at 4°C for 21 days (Aulisio et al., 1980; Velázquez et al., 1996; Singh et al., 2003). After 7, 14 and 21 days of incubation, alkaline post enrichment treatment was performed by homogenizing 0.5 ml of enriched samples with 4.5 ml of 0.5% KOH in 0.5% NaCl for 30 seconds as described by Siriken (2004), followed by spread plating on Cefsulodin-Irgasan-Novobiocin agar (CIN agar, Oxoid CM 653 containing selective supplement SR 109) and incubation at 30°C for 24 h (FDA, 2001). Typical small (1-2 mm diameter) "bull's eye" colonies having deep red center with sharp border, surrounded by clear colourless zone with entire edge were isolated and inoculated into Lysine Arginine Iron Agar (LAIA) slants. Isolates showing alkaline slant and acid butt without any gas or H_2S formation in LAIA slants and urease-positive reaction were considered as presumptive *Yersinia* species. All presumptive isolates were confirmed using biochemical tests prescribed by FDA (2001) and Bottone et al. (2005). The API 20E system (Bio Merieux, France) was employed for the confirmation of the *Yersinia* isolates.

The identified *Y. enterocolitica* isolates were biotyped and phenotypic virulence tests such as temperature dependent autoagglutination, salicin fermentation, aesculin hydrolysis, calcium dependent growth and congo red absorption and pyrazinamidase production were performed (Bhaduri, 1994; FDA 2001). The serogroups O3, O5, O8 and O9 of Wauters' classification (Wauters, 1981) of *Y. enterocolitica* were determined by slide agglutination using available antisera (Denka and Seiken Co Ltd. Tokyo, Japan).

Antibiotic susceptibility

The susceptibility of *Y. enterocolitica* isolates and reference strain ATCC 23715 to antibiotics was assessed by the agar diffusion method of Bauer et al.(1966) using Mueller Hinton agar (Difco, 225250, USA) as a test medium and commercially available antibiotic test discs: Ampicillin (10 µg), Carbenicillin (100 µg), Cephalothin (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (1 µg), Colistin (10 µg), Gentamycin (10 µg), Kanamycin (30 µg), Nalidixic acid (30 µg), Streptomycin (10 µg), Sulphamethizole (300 µg), Tetracycline (30 µg) and Trimethoprim (25 µg) (Hi-Media, India). Test plates were incubated for 16 to 18 h at 35°C under aerobic conditions. Results were interpreted according to National Committee for Clinical Laboratory Standards (NCCLS, 2003; 2005). For quality control, Mueller Hinton agar was tested with *Escherichia coli* ATCC 25922.

Detection of the genes *inv*, *ail* and *yst* by PCR

Bacterial DNA was prepared by the phenol-chloroform method as described by Sambrook et al. (1989). The general PCR procedure was performed according to the method of Lambertz & Danielsson-Tham (2005).

The primer pairs (5′AAT GCT GTC TTC ATT TGG AGC 3′ and 5′ATC CCA ATC ACT ACT GAC TTC 3′) for detection of the *yst* gene (a heat stable enterotoxin of *Yersinia*) were amplified a 145bp DNA fragment from the chromosome (Ibrahim et al., 1997). The primer pairs (5′- GTT TAT CAA TTG CGT CTG TTA ATG TGT ACG - 3′ and 5′- CTA TCG AGT TTG GAG TAT TCA TAT GAA GCG -3′) for detection of the ail gene (attachment and invasion

locus) amplified a 454 bp DNA fragment from the chromosome (Lambertz et al., 2000). The primer pairs (5′- CTG TGG GGA GAG TGG GGA AGT TTG G - 3′ and 5′- GAA CTG CTT GAA TCC CTG AAA ACC G -3′) for detection of the *inv* gene (cell invasion), amplified a 570 bp DNA fragment from the chromosome (Falcão et al., 2004). PCR reactions were performed in a final volume of 25 µl. The reaction mix contained a final concentration of 200 µM of each of the four dinucleotide triphosphates, 3 mM $MgCl_2$, 1 µM of each of the six primers, 1unit of Taq DNA Polymerase (Fermentas, Germany) and 1 µl of total *Y. enterocolitica* DNA. PCR was performed for 30 cycles of 3 min at 94°C (for initial denaturation) in Mastercycler Personnel (Eppendorf, Germany) followed by 30 cycles of 30 sec at 94°C (for denaturation of template DNA), 1 min at 60°C (for annealing), 1 min at 72°C (for extension) and 5 min at 72°C (for final extension). The PCR products were separated in 1.5% agarose gel and documented by a gel documentation system (AlphaImager, AlphaInnotech Corporation, California) after staining the gel with ethidium bromide (0.5 µg ml^{-1}). The *Y. enterocolitica* type culture (ATCC 23715) containing all the tested genes was used as positive control. Reaction mixture without DNA template was used as negative control.

Results and Discussion

Isolation of *Yersinia* spp.

Of the thirty finfish and shellfish samples examined, *Y. enterocolitica* was detected in one sample (Table 1, 2). Sixty three *Yersinia* strains were recovered from 70-80% of the finfish/shellfish, of which 54% corresponded to *Y. intermedia*, 19% to *Y. aldovae*, 10% *to Y. rohdei*, 5% to *Y. bercovieri*, 5% to *Y. enterocolitica*, 3% to *Y. kristensenii*, 2% to *Y. pseudotuberculosis* and 2% to *Y. frederiksenii* (Table 3). Khare et al. (1996) reported dominance of *Y. intermedia* with isolation frequency of 54% and *Y. enterocolitica* (4%) in fish and shellfish from retail markets in Mumbai (India). However, low numbers of *Y. intermedia* and high number of *Y. enetrocolitica* were recorded in Australia (Ibrahim & Mac Rae, 1991) from red meat and milk.

All the four *Y. enterocolitica* isolates from shellfish were biotyped as biotype 1A whereas the reference strain (ATCC 23715) belonged to 1B (Table 4). The four *Y. enterocolitica* strains did not belong to pathogenic serogroups O3, O5, O8 and O9. Biotype 1A was reported in water and sewage samples from Tamil Nadu, Delhi and Agra in India (Abraham et al., 1997, Sinha et al., 2000). Sinha et al. (2000) recovered biotype 1A belonging to serogroups O-6, 30-6, 31,

Table 1. Incidence of *Yersinia* spp. in finfish species collected from south-west coast of India

Sample analysed	No. of samples tested	No. of *Yersinia* spp. isolated	No. of samples positive for *Y. enterocolitica*
Marine fish	1	ND	ND*
Nemipterus japonicus			
Otolithus spp.	1	2	ND
Rastrelliger kanagurta	1	2	ND
Sardinella longiceps	1	ND	ND
Trichiurus lepturus	1	13	ND
Brackishwater fish	2	ND	ND
Chanos chanos			
Etroplus suratensis	1	1	ND
Mugil cephalus	1	2	ND
Freshwater fish			
Labeo rohita	2	1	ND
Tor tor	4	1	ND
Oncorhynchus mykiss	2	5	ND
Pangasianodon hypophthalmus	1	ND	ND

*Not Detected

Table 2. Incidence of *Yersinia* spp. in shellfish species collected from south-west coast of India

Sample analysed	No. of sample tested	No. of *Yersinia* spp. isolated	No. of sample positive for *Y. enterocolitica*
Metapenaeus dobsoni	1	1	ND*
Fenneropenaeus indicus	1	3	ND
Penaeus monodon	1	ND	ND
Scylla serrata	2	4	ND
Villorita cyprinoides	1	ND	ND
Villorita cyprinoides (Boiled)	1	1	ND
Sepia spp.	1	5	ND
Loligo duvauceli	3	18	1+**
Octopus membranaceus	1	3	ND

*Not Detected + **-sample positive

Table 3. Distribution of *Yersinia* spp. (%) in finfish and shellfish species collected from south-west coast of India

Samples	% distribution of *Yersinia* spp.								
	Total. isolates	*Y. intermedia*	*Y. aldovae*	*Y. bercovieri*	*Y. pseudo-tuberculosis*	*Y. kristen-senii*	*Y. entero-colitica*	*Y. frederi-ksenii*	*Y. rohdei*
Rastrelliger kanagurta	2	100	-	-	-	-	-	-	-
Otolithus spp.	2	–	-	-	-	-	-	-	100
Trichiurus lepturus	14	57.1	42.9	-	-	-	—	-	-
Etroplus suratensis	1	100	-	-	-	-	-	-	-
Mugil cephalus	2	–	-	50	-	-	-	-	50
Labeo rohita	1	100	-	-	-	-	-	-	-
Oncorhynchus mykiss	5	80	20	-	-	-	-	-	-
Tor tor	1	–	-	-	-	-	-	-	100
Fenneropenaeus indicus	3	6.7	–	–	–	33.3	–	–	–
Metapenaeus dobsonii	1	100	–	–	–	–	–	–	–
Scylla serrata	4	100	–	–	–	–	–	–	–
Villorita cyprinoides	1	100	–	–	–	–	–	–	–
Loligo duvauceli	18	33.3	16.6	5.6	5.6	5.6	22.2	–	11.1
Sepia spp.	5	60	–	20	–	–	–	20	–
Octopus membranaceus	3	100	–	–	–	–	–	–	–

O:10-34, O:6-31, O:15, O:41,42 and O:41,43 from wastewater in India. *Y. enterocolitica* serotype O:6,30 belonging to biotype 1A isolated from diarrhoetic patients in Delhi as reported by Singh et al. (2003) has also been implicated in nosocomial and milk-borne outbreaks in certain parts of the world (Ratnam et al., 1982; Butt et al., 1991).

All the *Y. enterocolitica* isolates showed no virulence related phenotypes with respect to esculin hydrolysis, salicin fermentation and pyrazinamidase production. All the isolates were negative for autoagglutination test, calcium dependence at 37°C and congo red absorption on CR-BHO agarose, indicating that the isolates did not carry the virulence plasmid.

Antibiotic resistance profile

The tests of antibiotic resistance showed that all the *Y. enterocolitica* strains and reference strain were resistant (100%) to ampicillin, cephalothin and

Table 4. Biotyping* of *Y. enterocolitica* strains isolated from squid

Y. enterocolitica Strain	Lipase	Esculin	Salicin	Indole	Xylose	Trehalose	Pyrazina-midase	β-D-gluco-sidase	Voges - Proskauer (VP)	Biotype
ATCC 23715	+	–	–	+	+	+	-	-	+	1B
YE 1	+	+	+	+	+	+	+	+	+	1A
YE 2	+	+	+	+	+	+	+	+	+	1A
YE 3	+	+	+	+	+	+	+	+	+	1A
YE 4	+	+	+	+	+	+	+	+	+	1A

*Based on Bottone et al. (2005), FDA (2001)

sulphamethizole (Table 5). In the present study, none of the strains were resistant to carbenicillin, chloramphenicol, ciprofloxacin, colistin, gentamycin, nalidixic acid, tetracycline and trimethoprim. These findings are in accordance with previous studies (Kwaga & Iversen, 1990; Funk et al. 2000; Baumgartner et al. 2007; Divya & Varadaraj 2011). The resistance profile obtained is in agreement with previous studies in which resistance to ampicillin and many cephalosporins were frequently observed (Funk et al., 2000; Lee et al., 2004, Baumgartner et al., 2007).

Table 5. Antimicrobial resistance of *Yersinia enterocolitica* Biotype 1 A isolated from squid

Antimicrobial agent	*Yersinia enterocolitica* % resistance	
	ATCC 23715 Biotype 1B	isolates from shellfish (n=4) Biotype 1A
Ampicillin	100	100
Cephalothin	100	100
Carbenicillin	0	0
Chloramphenicol	0	0
Ciprofloxacin	0	0
Colistin	0	0
Gentamycin	0	0
Kanamycin	0	0
Nalidixic acid	0	0
Streptomycin	0	0
Sulphamethizole	100	100
Tetracycline	0	0
Trimethoprim	0	0

Detection of virulence genes

The virulent genes *ail* and *yst* were not detected in *Y. enterocolitica* biotype 1A. The *inv* gene was detected in all the strains studied (Fig. 1). The *ail*, *inv* and *yst* genes were detected in reference strain biotype 1B. The lack of the virulence markers *ail* and *yst* in *Y. enterocolitica* biotype 1A isolates in the present study confirmed earlier studies (Miller et al., 1989; Pierson & Falkow, 1990; Bhagat & Virdi, 2007). The *ail* gene was reported in *Y. enterocolitica* biotype 1A strains, which are commonly considered as nonpathogenic (Kraushaar et al., 2011). Grant et al. (1998) reported pathogenic biotype 1A strains that lack yst gene. Tennant et al. (2003) have suggested that there are two subgroups of biotype 1A of *Y. enterocolitica*: a group comprising pathogenic strains of clinical origin and another group comprising non-pathogenic strains of environmental origin. It has been suggested that biotype 1A of *Y. enterocolitica*

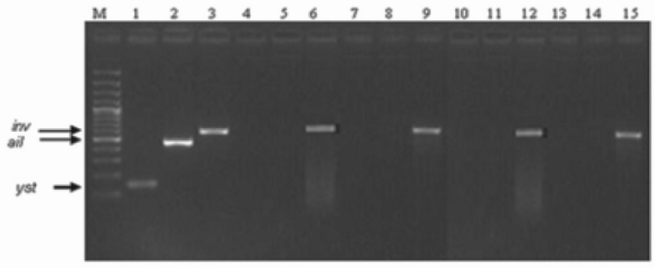

Fig. 1. PCR detection of genes *yst* (145bp), *ail* (454bp) and *inv* (570bp) encoding virulence –associated properties in *Y. enterocolitica* isolates from shellfish

Lanes 1, 2 and 3 *Y. enterocolitica* type strain no. 23715; Lanes 4, 5 and 6 *Y. enterocolitica* isolate YE 1; Lane 7, 8 and 9 YE 2; Lanes 10, 11 and 12 YE 3; Lanes 13, 14 and 15 YE 4; Lane M: 100 bp ladder as the DNA size control (Fermentas, Germany).

may be pathogenic by some novel, as yet undetermined mechanisms (Bottone, 1999). Isolates of biotype 1A have constituted a sizeable fraction of isolates from patients with gastroenteritis (Burnens et al., 1996; Robins-Browne, 2001). In an outbreak of gastroenteritis in Canada involving nine patients, the causative agent was *Y. enterocolitica* biotype 1A, serogroup O:5 (Ratnam et al., 1982). Falcão et al. (2006) reported presence of virulent genes *ail* and *yst* A in biotype 1A / O:10 strain isolated from food. Although few, there are some published data showing the involvement of *Y. enterocolitica* 1A/O : 10 in human infection (Greenwood & Hooper, 1990; Morris et al., 1991). This result point to its pathogenic potential and suggests that food contaminated with *Y. enterocolitica* biotype 1A may cause infection.

In conclusion, the present investigation revealed presence of *Y. enterocolitica* biotype 1A in squid from India. These biotype 1A isolates did not belong to pathogenic serogroups O:3, O:5, O:8 and O:9 and showed resistance (100%) to ampicillin and cephalothin. The antimicrobial resistance in these Y. *enterocolitica* biotype 1A isolates is an issue of concern because these isolates can transfer antibiotic resistance genes to the pathogenic strains. Recent reports of the isolation of nonpathogenic strain in association with clinical illness should prompt continued efforts to define biotypes and serogroups of *Y. enterocolitica* strains which may cause diseases through alternative pathogenic mechanisms. Due to the psychrotrophic nature and the importance of *Y. enterocolitica* as a potential food- and water-borne enteric pathogen, there is a need to perform microbiological controls of fish and fishery products to reduce consumer risks.

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