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Research Note

Prevalence and Characterization of Typical and Atypical *Escherichia coli* from Fish Sold at Retail in Cochin, India

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ABSTRACT

Escherichia coli is a common contaminant of seafood in the tropics and is often encountered in high numbers. The count of *E. coli* as well as verotoxigenic *E. coli* O157:H7 was estimated in 414 finfish samples composed of 23 species of fresh fish from retail markets and frozen fish from cold storage outlets in and around Cochin, India. A total of 484 presumptive *E. coli* were isolated, and their indole–methyl red–Voges-Proskauer–citrate (IMViC) pattern was determined. These strains were also tested for labile toxin production by a reverse passive latex agglutination method and checked for *E. coli* serotype O157 by latex agglutination with O157-specific antisera. Certain biochemical marker tests, such as methylumbelliferyl-β-glucuronide (MUG), sorbitol fermentation, decarboxylase reactions, and hemolysis, which are useful for screening pathogenic *E. coli,* were also carried out. Results showed that 81.4% of the *E. coli* isolates were sorbitol positive. Among this group, 82% were MUG positive, and 14.46% of the total *E. coli* isolates showed human blood hemolysis. None of the isolates were positive for agglutination with *E. coli* O157 antisera nor did any produce heat-labile enterotoxin. This study indicates that typical *E. coli* O157 or labile toxin–producing *E. coli* is absent in the fish and fishery environments of Cochin (India). However, the presence of MUG and sorbitol-negative strains that are also hemolytic indicates the existence of aberrant strains, which require further investigation.

Foodborne diseases are one of the most widespread health problems in the contemporary world, and with the vast expanse in international trading and globalization, there is an increased urgency for ensuring the quality of the food that is marketed. Apart from well-known pathogenic microorganisms such as *Salmonella* and *Vibrio cholerae,* several emerging pathogens have been recognized in recent years, including enteropathogenic and verotoxigenic *Escherichia coli.* According to the report of the World Health Organization, *E. coli* is the leading cause of food poisoning outbreaks in developing nations *(24).*

Although beef is considered the main reservoir of verotoxin-producing *E. coli,* a retail meat study conducted by Doyle and Schoeni *(7)* isolated *E. coli* O157:H7 from 3.7% of beef, 1.5% of pork, 1.5% of poultry, and 2.0% of lamb samples tested, which indicates that the organism is associated with foods of other animal origin. A wide variety of food products, including unpasteurized apple cider, unpasteurized milk, raw potatoes, mayonnaise *(23),* and fermented sausage *(4),* also have been implicated in outbreaks due to *E. coli* O157:H7. There are few reports on the occurrence of pathogenic strains of *E. coli* in seafood from India or other parts of the world *(19, 27, 30),* although *E. coli* is a major contaminant of seafood *(25, 29).* As reported by the Food and Agriculture Organization (FAO), there is not yet, to our knowledge, any report of isolation of *E. coli* O157:H7 from seafood products *(16).* However, one outbreak due to the consumption of fish contaminated with *E. coli* O157 has been reported *(6).*

The objectives of this study were to estimate the presence of toxigenic *E. coli* among the natural *E. coli* populations in seafood in and around Cochin, India, and to evaluate the immediate threat to public health and safety from seafood consumption.

MATERIALS AND METHODS

Collection of fish samples. A total of 414 samples of finfish consisting of 280 raw unprocessed fish collected from open markets and 134 frozen fish collected from cold storage establishments in and around Cochin, India, for 6 years (from April 1996 to October 2002) were analyzed. Altogether, 23 fish species from marine, brackish, and freshwater sources were collected during this study period. The samples were brought to the laboratory aseptically and analyzed immediately. These fish represented the most readily available species in this area.

Enumeration and isolation of *E. coli* strains. *E. coli* counts of the fish samples were estimated by a three-tube most-probable-number (MPN) procedure *(31)* with the following modification. Ten grams of the muscle portion of fish along with skin was homogenized for 1 min with 90 ml of saline (0.85% NaCl) in a stomacher 400 lab blender (Seward, London, UK). Aliquots of serially diluted samples were inoculated into MacConkey broth (Oxoid, Basingstoke, UK) and incubated at 37°C for 24 to 48 h. Positive tubes were (i) subjected to an MPN procedure in brilliant green lactose bile broth (Oxoid) at 37°C for 24 to 48 h and (ii) subjected to elevated coliform (Difco, Becton Dickinson, Sparks,

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TABLE 1. *Prevalence of* Escherichia coli *in raw and frozen fish from retail outlets in Cochin, India, and their phenotypic traits for toxigenicity*

Fish	No. of samples tested	*E. coli* count (MPN/g) (range)[a]	Total isolates	No. of positives			
				Sorbitol	Labile toxin	Latex	β-hemolysis
Raw *Rastrelliger kanagurta* (Indian mackerel)	22	0.4–140+	22	22	0	0	7
Raw *Parastromateus niger* (silver pomfret)	17	0–140+	18	13	0	1[b]	0
Raw *Scomberomorus commerson* (seerfish)	31	0.4–1,100	39	29	0	0	0
Raw *Etroplus suratensis* (pearlspot)	11	15–1,400	15	11	0	0	6
Raw *Lethrinus fraenatus* (pig face emperor)	16	0.9–140+	20	17	0	0	0
Raw *Lutjanus argentimaculatus* (red snapper)	31	0.4–140+	37	28	0	0	0
Other raw fish[c]	152	0–140+	169	142	0	6[b]	24
Frozen *P. niger* (silver pomfret)	15	2.5–140+	14	12	0	0	0
Frozen *S. commerson* (seerfish)	9	0.4–140+	12	10	0	0	2
Frozen *R. kanagurta* (Indian mackerel)	14	0–140+	19	15	0	0	0
Frozen *E. suratensis* (pearlspot)	17	0–140+	24	19	0	0	1
Frozen *L. fraenatus* (pig face emperor)	11	4.5–140+	15	14	0	0	0
Froze *Acanthopagrus berda* (sea bream)	8	4.5–140+	10	8	0	0	2
Other frozen fish[c]	60	0–140+	70	54	0	0	28
Total	414	—	484	394 (81.4%)	0	7[b] (1.44%)	70 (14.46%)

[a] Average count could not be expressed, as some of the values were 140+.
[b] Self-agglutination observed.
[c] Includes croaker, reef cod, sardine, pink perch, tuna, barracuda, tilapia, rohu, snappers, carangids, and silver bellies.

Md.) and indole (Difco, Becton Dickinson) broths at 44.5°C, and counts were estimated according to the MPN table. A loopful of growth from indole-positive tubes was streaked on eosine methylene blue agar (BBL, Becton Dickinson, Sparks, Md.), and characteristic *E. coli* colonies were isolated and confirmed by indole–methyl red–Voges-Proskauer–citrate (IMViC) tests. Representative cultures (5%) were cross-checked for identification status by bioMérieux API 20E (bioMérieux, Inc., Marcy l'Etoile, France). Further, these *E. coli* isolates were screened for sorbitol fermentation and checked for the presence of methylumbelliferyl-β-glucuronide (MUG) enzyme activity by inoculating them onto violet red bile glucose agar (Difco, Becton Dickinson) supplemented with MUG (BR071E, Oxoid) to a final concentration of 100 mg/liter. After incubation at 37°C for 24 h, fluorescent colonies were recorded under a UV lamp.

For the detection of *E. coli* O157:H7, 25 g of the fish flesh with external skin was enriched in 225 ml of modified elevated coliform broth (Difco, Becton Dickinson) containing novobiocin (20 μg/ml; Sigma Chemical Company, St. Louis, Mo.) at 37°C with shaking (150 rpm) for 20 to 24 h. After an overnight incubation, diluted enrichment samples were plated on MacConkey sorbitol agar (Oxoid) *(22)* supplemented with cefixime (SR 0191, Oxoid) and potassium tellurite *(33).* Plates were incubated overnight at 42°C, and sorbitol-negative colonies were isolated at a rate of two to three colonies per sample. After isolate purification, they were streaked on eosine methylene blue agar, and confirmed isolates were checked for MUG reaction and IMViC test. MUG and sorbitol-positive *E. coli* (ATCC 25922) were used as controls for checking sorbitol and MUG reactions. Isolates that were sorbitol and MUG negative were tentatively grouped as *E. coli* O157:H7.

Detection of toxigenic *E. coli* and *E. coli* O157:H7. All *E. coli* isolates were tested using the VET (*V. cholerae* enterotoxin) reverse passive latex agglutination kit (TD 0920, Oxoid) for heat-labile enterotoxin production according to the procedure outlined by the manufacturers. Isolates that were sorbitol negative and MUG negative were tested for latex agglutination with *E. coli* serotype O157-specific antisera (DR 620M, Oxoid). The isolates were grown on brain heart infusion (Oxoid) slants overnight and tested for latex agglutination according to the manufacturer's instructions. *E. coli* O157:H7 (ATCC 43895) was used as a positive control. All of the strains were also tested for lysine and ornithine decarboxylase *(21),* and beta-hemolytic activity was studied in nutrient agar with 5% human erythrocytes that had been received from State Health Authorities (Cochin).

RESULTS AND DISCUSSION

Fish samples from different retail markets were analyzed for the prevalence of *E. coli,* and the data are presented in Table 1. Altogether, 484 *E. coli* isolates were obtained during the study, accounting for both sorbitol-negative and sorbitol-positive strains. The prevalence of *E. coli* was consistently high and was above the acceptable limit of 20 CFU/g for fish in most cases (66% of the total samples analyzed). The count showed wide fluctuation and varied from 0 to 10^3 MPN/g. All of the isolates in this study were eosine methylene blue positive and thus conformed to presumptive *E. coli.* A stable property of verotoxigenic *E. coli* is its inability to ferment sorbitol, and this trait has been made use of in designing media for its detection *(22).* It was observed in the present investigation that 81.4% of the isolates were sorbitol positive. Among clinical isolates, >95% are reported to be sorbitol positive *(32).*

The isolates were further grouped on the basis of IMViC reaction. Geldreich *(12)* has described three types of *E. coli* on the basis of their IMViC pattern (Table 2). In both sorbitol-positive and sorbitol-negative isolates, most of the isolates were biotype I (+ + − −), but there was a

TABLE 2. *Differentiation of presumptive* Escherichia coli *isolates on the basis of their IMViC pattern*

IMViC type	% sorbitol negative (n = 90)	% sorbitol positive (n = 394)
Biotype I (++−−)	50	92
Biotype II (−+−−)	6	0
Fecal type (+−−−)	24	3
Other types	20	5

TABLE 3. *Differentiation of sorbitol-negative and -positive* Escherichia coli *on the basis of their phenotypic traits*

Biochemical traits	% sorbitol negative (n = 90)	% sorbitol positive (n = 394)
MUG positive	43	82
Lysine decarboxylase positive	16	72
Ornithine decarboxylase positive	34	80
Hemolytic	44	7

higher percentage of this group among sorbitol-positive strains (92%) than among sorbitol-negative strains (50%). Fecal type (+ − − −) was generally more sorbitol negative (24%) than positive (3%). This indicated a possibility that verotoxigenic *E. coli*, which is sorbitol negative, is an atypical *E. coli* type.

A negative reaction to MUG is exhibited by verotoxigenic *E. coli (7).* Almost 82% of the sorbitol-positive isolates were MUG positive, while only 43% of the sorbitol-negative isolates showed MUG activity. There are reports that >94 to 96% of *E. coli* isolates from clinical sources were positive for glucuronidase activity *(8, 10, 17).* However Chang et al. *(5)* reported that only 65% of human fecal *E. coli* isolates were β-D-glucuronidase positive. Lum and Chang *(20)* also reported that 66% of the *E. coli* isolates from human fecal sources and 69% from animal fecal sources were β-D-glucuronidase positive, while 100% of the clinical strains were β-D-glucuronidase positive. While confirming the results of the authors, the present data show that a sizable fraction of sorbitol-negative isolates are also MUG positive.

Haldane et al. *(14)* have marketed supplementary tests such as lysine and ornithine decarboxylase as biochemical markers to improve the specificity of sorbitol screening procedure. According to the above authors, verotoxigenic *E. coli* exhibits lysine-negative and ornithine-negative patterns. In the present study, 72% of the sorbitol-positive isolates were lysine positive, and 80% were ornithine positive (Table 3). On the contrary, 16% of the sorbitol-negative isolates were lysine positive, and 34% were ornithine positive. There is a small fraction of *E. coli* with positive lysine and ornithine decarboxylase activity among sorbitol-negative *E. coli*, which points to the limitation of biochemical traits for the identification of verotoxigenic *E. coli.*

A rapid screening procedure that is based on hemolytic activity is also reported for differentiating pathogenic hemolytic strains of *E. coli (2).* In the present study, 14.46% of the total isolates showed the beta-hemolyzing activity (Table 3). The hemolytic activity was more prevalent among sorbitol-negative isolates (44%) than among sorbitol-positive isolates (7%). The data confirm that hemolytic activity is common to both sorbitol-negative and sorbitol-positive isolates, though there is a greater prevalence among sorbitol-negative isolates.

The rapid latex test marketed by Oxoid is a reliable method for detecting *E. coli* serotype O157, as supported by previous data *(26).* None of the isolates, whether of typical or atypical phenotype, gave a positive latex agglutination reaction (Table 1). False agglutination reactions were noted in a limited number of strains, and this was observed with isolates from croaker, *Otolithus cuvieri*, and reef cod, *Epinephelus malanostigma.* According to Borczyk et al. *(3),* false-positive agglutination occurs with several sorbitol-negative *Escherichia* spp., including *E. hermanii, E. coli* O148:NM, and *E. coli* O117:H27. There are also reports of the occurrence of atypical *E. coli* O157:H7, which fails to show the typical biochemical traits *(9, 13, 15).*

There are reports on the Shiga-like toxin–producing *E. coli* from seafood *(19, 27).* The present study failed to detect *E. coli* O157 labile toxin–producing *E. coli.* Earlier attempts by Adesiyun *(1),* Fernandes et al. *(11),* and Surendran et al. *(28)* that investigated different fish sources in Trinidad, the United States, and Cochin reported similar results. Kokubo et al. *(18)* detected labile toxin in *E. coli* isolates from oyster.

A small portion (i.e., 39 of 484 *E. coli* isolates) exhibited all the traits for enterohemorrhagic *E. coli* O157:H7, except for latex agglutination, the major distinguishing criteria for detection. The results indicate that typical verotoxigenic *E. coli* O157 or enterotoxigenic types of *E. coli* are absent in seafood from this area. At the same time, hemolytic types with many of the accessory traits attributable to verotoxigenic types are noted. This suggests the presence of atypical strains that require further study at the molecular level. It is inferred that although *E. coli* is high in numbers in seafood from Cochin (India), typical *E. coli* O157 could not be isolated.

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