ORIGINAL ARTICLE

Detection of *Yersinia enterocolitica* in diarrhoiec stools and environmental samples

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ABSTRACT

Aim: To detect distribution of *Yersinia enterocolitica* in gastroenteritis cases, in environmental samples and food samples consumed by humans. **Materials and Methods:** Stool samples, environmental samples comprising water from water pools on the bank of river 'Kabini,' fruit juices from commercial fresh juice centers, pork intestinal contents, drinking water from public eateries, ('paani puri' outlets), public tap water samples and commercial prepacked juices. All samples were cultured by four different methods; direct plating on MacConkey agar, plating following cold enrichment, single alkali and double alkali treatment with KOH & PBS. **Results:** A total of 181 stool samples and 15 food and environmental samples were processed. *Y. enterocolitica* was isolated from one stool sample (0.55%) by cold enrichment method and from one water sample collected from river bank (6.6%). **Conclusion:** *Y. enterocolitica* is found in humans and in the environment in this region. Adopting a rapid isolation method may infuse interest among clinical microbiologists to undertake an epidemiological study on *Y. enterocolitica* and Yersiniosis. Unhygienic defaecation habits of the people and movement of swine herds along the river bank may be the source for river water contamination.

Key words: Cold enrichment, chitterling, Yersiniosis, Zoonotic

INTRODUCTION

Y. enterocolitica is a human pathogen that is ubiquitous in livestock; especially pigs.[1] It was named by Friderichsen. Schleifstein and Coleman first described *Y. enterocolitica* in 1939.[2] Further interest in this bacterium developed only during the 1970s when the method of isolation was defined. Yersiniosis is a food-borne bacterial zoonotic disease transmitted mainly by consumption of undercooked or raw chitterlings.

Vegetables and untreated water may also serve as source of infection. Occasionally, pathogenic *Y. enterocolitica* has been detected in vegetables and environmental water.[3] Extensive isolation studies may detect other refrigerated food items also as the source.[4] However, the isolation rates of pathogenic *Y. enterocolitica* have been low, which may be due to the low sensitivity of the present detection methods.[5]

Y. enterocolitica is being more frequently detected both from environment and human sources. Apart from intestinal infections, it is also known to cause non-purulent arthritis, sepsis and erythema nodosum.

This study aims to find out the distribution of *Y. enterocolitica* among the cases of gastroenteritis admitted in JSS Hospital, Mysore, in food samples like fruit juices, intestinal content and muscle of swine and also in environmental samples like river water and water used in public eateries at Mysore, Karnataka. This study also evaluated four different isolation methods to find the most sensitive culture method for *Y. enterocolitica*.

MATERIALS AND METHODS

A one-year study during January 2006 to December 2006 was undertaken. The study proposal was submitted to the Ethical clearance committee of JSS hospital and approved.

Stool samples

Inclusion criteria

Stool samples in which other cause(s) of gastroenteritis was not detected.

Exclusion criterion

Stool samples in which another cause has been demonstrated.

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Gastroenteritis cases from both children and adults hailing from rural and urban areas attending the outpatient department and cases admitted in medicine and paediatric unit were chosen. The significance of the study, publication in a peer-reviewed journal and maintenance of confidentiality was explained in the local language to parents of children enrolled in the study. Adults who volunteered to participate were similarly briefed about the study and a written consent was collected from the adult patients and the parents of the child participants. Commercial establishment owners were told in detail about the study and requested to cooperate to collect appropriate samples. A voluntary written consent was obtained from them. The ethical committee approved the method of sample collection and the ethical issue and granted permission to proceed with the study. No invasive procedure was undertaken during the study.

Water from river and municipal taps, fruit juices from juice centers, intestinal contents of pork, water used in 'pani puri' outlets and commercial fruit juices in cartons were the main environmental and food samples.

Sample collection

Stool samples were collected in sterile disposable wide-mouthed containers without any admixture of urine. The environmental samples were collected in sterile containers. A total volume of 100 ml of each sample was collected. Perfect cold chain was maintained during the transportation of test samples.

Sample processing

Stool samples

All diarrhoiec stool samples were subjected to:

a. Direct microscopic examination - to detect the presence of inflammation and parasites or their ova. All stool samples were subjected to preliminary microscopic examination using saline and iodine wet mount preparation to detect parasites, RBC and WBC.
b. Immediate direct culture was done on MacConkey agar plate, XLD agar and DCA plate to detect *Y. enterocolitica* and/or other enteric pathogens.
c. Three different enrichment methods for selective isolation of *Y. enterocolitica* were tried.
    1. Cold enrichment - about 2g of stool was mixed with 5 ml of PBS (pH 7.4) and incubated at 4°C.[6,7]
    2. Alkali enrichment method - stool sample was mixed with 0.5% KOH in the ratio of 1:2 for 2 min.[6,7]
    3. Double enrichment - after alkali treatment, sample was transferred to 5 ml PBS and incubated at 4°C for 24 hours.

Environmental samples

1. Water: 50 ml of water sample was mixed with 450 ml cold enrichment broth and incubated at 4°C for 3 weeks.[8]
2. Commercial fruit juice in cartons, 'pani puri' water, fresh fruit juices were processed similar to water.
3. Intestinal content of pork: About 2 g of intestinal content was mixed with 5 ml of PBS and incubated at 4°C for 2 weeks (ph 7.4).[9]
4. Pork: About 25 g of thigh muscle was homogenized in a blender. And to this 225 ml of PBS mixed with 1.5% bile salt was added and incubated at 4°C for 2 weeks.[9]

After the preliminary enrichment, all the samples were further processed uniformly as follows.

Subcultures were made on two sets of *Y. enterocolitica* CIN agar and MacConkey agar. One set of plates was incubated at 37°C and other set at 22°C for 24 hours.

Tiny NLF colonies on MacConkey and colonies having bulls-eye appearance on CIN agar were further subjected to the identification tests described below and confirmed as *Y. enterocolitic*a.[10]

Gram staining-Gram negative bacilli, Differential motility — Non-motile at 37°C and motile at 22°C, Catalase — positive, Oxidase — Negative, Indole positive, Urease positive, Citrate negative, TSI -A/A Arginine and Lysine not decarboxylated, Ornithine decarboxylated, Rhamnose not fermented, Raffinose not fermented.

The isolates showing the above results was recognized as *Y. enterocolitica* presumptively. An isolate not showing the above reactions was identified as other enteric pathogen and was further confirmed by testing specific biochemical identification tests and by slide agglutination employing specific anti-sera. (All the isolates were preserved for further reference).

RESULTS

This study aimed only at detection of *Y. enterocolitica*, from stool and environmental samples. The statistical operations were done through SPSS for Windows SPSS Inc., New York. Total 181 cases of enteritis diagnosed at JSS hospital Mysore and 15 environmental samples from different sources were studied [Table 1].

Presence of WBCs was seen in 103 samples of which 43 showed presence of RBC also. Parasitic forms were found in 10 out of 181 samples, which included *G. intestinalis* (06), eggs of *Hookworm* (01), eggs of *Ascaris lumbricoides* (02), and *Entamoeba histolytica* (01) [Table 2].

None of the 181 stool samples yielded growth of *Y. enterocolitica* by direct plating method [Table 3].

Cold enrichment and plating on to MacConkey agar suppressed the growth of enteric pathogens. One stool sample out of 181 yielded the growth of *Y. enterocolitica* on MacConkey agar and also on CIN agar after cold enrichment (0.5%). During the initial study period lasting for two months between January and March 2006, 60 stool samples were subjected to two cold enrichment methods only. Using PBS and 0.5% KOH enrichment methods after incubation at 4°C for 7 days, one stool sample from a child aged one year yielded the growth of *Y. enterocolitica*. The other 59 samples subjected to cold enrichment did not yield the growth of any pathogens. In order to increase the isolation rate of *Y. enterocolitica*, we employed the double enrichment method.

Out of 181 stool samples, total number of enteric pathogens isolated was 44, including one isolate of *Y. enterocolitica* (24.3%). The rest (137) yielded the growth of *Escherichia coli* and other intestinal commensal bacteria only [Table 4].

In the present study, an attempt was made to compare the efficacy of different enrichment techniques for isolation of *Y. enterocolitica* and to prevent the growth of other bacteria. It was found that PBS enrichment at 4°C for 7-14 days followed by plating on to MacConkey media yielded the growth of *Y. enterocolitica*. But this method yielded the growth of few commensal bacteria also. Alkali enrichment followed by plating as above was equally efficient in isolation of *Y. enterocolitica* isolation and in addition this technique was efficient in inhibiting the growth of other bacteria. Thus from the results of the present study it can be said that alkali enrichment may probably be superior to PBS alone for inhibiting the growth of intestinal commensals & other enteric pathogens.

In the present study 15 environmental samples from different sources were studied [Table 5].

All the 15 samples were subjected to cold enrichment using PBS alone. Among the 15 such samples included, one water sample of Kabini river, which was present as a stagnant water pool along the minor canal yielded growth of *Y. enterocolitica*. Surprisingly the other sample of the same river collected from downstream yielded growth of *Aeromonas* species. None of the swine samples yielded growth of *Y. enterocolitica* in the present study. An additional finding from public health point was isolation of *E. coli* from tap water, water used in 'pani puri' outlets, swine intestinal contents and ground pork thigh muscle. The public health engineering department should impose strict hygienic practices in these places.

A continuous ongoing study of environmental samples throughout the year may further help in knowing the distribution of *Y. enterocolitica* and other enteric pathogens in this area of Karnataka.

Table 1: Distribution of samples

Sample Type	Number
Stool samples	181
Environmental samples	
Commercial juice	02
Fresh fruit juice	01
Water of 'pani puri'	02
Tap water	04
River water	02
Intestinal content of swine	02
Pork thigh muscle	02
Total environmental samples	15
Total number of samples	196

CC = 242; $P < 0.046$ (s)

Table 2: Direct Microscopy results

Total	WBCs		RBCs		Parasitic forms	
	Present	Absent	Present	Absent	Present	Absent
181	103	78	43	138	10	171
χ^2	3.453		49.862		143.21	
P value	0.063 (NS)		0.000 (HS)		0.000 (S)	

Table 3: Direct plating results

Bacterial isolates	Number
Shigella species	29
Vibrio cholerae	14
E. coli & others	137
Aeromonas	00

$\chi^2 = 150.10$; $P < 0.000$ (HS)

DISCUSSION

Yersiniosis is not a very common disease in humans. Occurrence is always related to inefficient food processing. CDC annual estimation is 1 case per 100,000 populations.

Table 4: Age and enteric isolates

Age in years	Bacterial isolates				
	Y. enterocolitica	*Shigella*	*Vibrio*	*E. coli and others*	Total
0-10	01	21	11	95	128
11-20	Nil	05	nil	10	15
21-30	Nil	Nil	03	06	09
31-40	Nil	Nil	nil	05	05
41-50	Nil	02	nil	14	16
50 and above	Nil	01	nil	07	08
Total	01	29	14	137	181

CC = 0.30; $P < 0.266$ (NS)

Table 5: Environmental samples

Environment samples	Bacterial isolates						Total
	Y. e	*Shigella sp*	*VC*	*Aeromonas*	E coli & others	No growth	
Commercial fruit juice	Nil	Nil	nil	nil	nil	02	02
Fresh fruit juice	Nil	Nil	nil	nil	nil	01	01
Water of 'pani puri'	Nil	Nil	nil	nil	02	nil	02
River water	01	Nil	nil	01	nil	nil	02
Tap water	Nil	Nil	nil	nil	02	02	04
Swine intestinal contents	Nil	Nil	nil	nil	02	nil	02
Pork thigh muscle	Nil	Nil	nil	nil	02	nil	02

It is more common in children below 5 years. *Y. enterocolitica* isolation rate in gastroenteritis is 1.4 to 2.8%.[11]

Enteritis and enterocolitis are the commonest health hazard reported all over the world.[2] There are several infective causes for enteritis. Most of these cause self-limiting ailment, and a few may lead to complications.

Several microbes that were hitherto considered as harmless commensals have recently emerged as newer pathogens. Lack of awareness about such newer pathogens among the attending physician has resulted in a very casual attitude of these physicians during the case management that leads to indiscriminate usage of antibiotics and anti-parasitic drugs. One such agent is *Y. enterocolitica*. For a long time, it was considered as a rare agent of enteritis. This was because of the tedious process of isolation and identification. Interest in *Y. enterocolitica* as one of the most common agents of diarrheal disease began since the time the culture method and selective culture media was proposed by Scheiman in 1979.[12] Since then many countries like Japan, USA and few European countries have reported. Different sources of *Y. enterocolitica* cited are water, pork, ice cream, milk, etc. *Y. enterocolitica* has been found to contaminate canned foods. Rate of isolation of *Y. enterocolitica* from diarrheic stools in several other countries ranges from 2-5%. The rate of isolation of *Y. enterocolitica* in our study from diarrheic stool sample was 0.5%. Our study correlates well with Singh *et al* who reported 0.6% isolation rate[13] and Varghese *et al* who reported 0.2%.[14] A slightly higher prevalence rate has been reported by Onyemelukwe *et al*, Nigeria,[15] Lal *et al*., Ludhiana,[16] and Ram *et al*, Chandigarh[17] [Table 6]. Due to small sample size in the present study, age-sex distribution of *Y. enterocolitica* cannot be evaluated.

In the present study, *Y. enterocolitica* was not isolated from any of the 181 stool samples by conventional direct plating method. Similar observations were made by Greenwood *et al*. Ram *et al* isolated one strain by direct plating method.[24] Since the colonies appear very tiny in direct plating method even after 48 hours of incubation, it may be missed or overlooked or overgrown by commensal flora. Thus it can be stated that direct plating of stool sample for *Y. enterocolitica* may be less efficient.[17,19]

Table 6: *Yersinia enterocolitica* — isolation rate Indian studies

Study	Percentage of isolation (%)
Varghese *et al*, New Delhi	0.2
Singh *et al*, Delhi	0.6
Onyemelukwe *et al*, Nigeria	1.4
Lal *et al*, Ludhiana	2.05
Ram *et al*, Chandigarh	3
Singh *et al*, Delhi	3
Present study	0.5

To overcome the problem, cold enrichment methods have been adopted. Basic principle is that *Y. enterocolitica* has ability to survive and multiply at refrigeration temperature while other enteric pathogens and commensals do not multiply.

Thus, it has been established that cold enrichment using PBS (ph 7.4),[6] alkali (0.5% KOH)[6] and incubation at 4°C for 7-14 days followed by usage of selective media (CIN agar) will help in isolation of *Y. enterocolitica*. Using these techniques many have succeeded in isolating *Y. enterocolitica*. In the present study, *Y. enterocolitica* was isolated by alkali enrichment too.

Y. enterocolitica is known to occur sporadically and also as outbreaks. Many investigators have reported the existence of *Y. enterocolitica* in environmental samples like water, chitterlings,[20] ice cream,[21] etc. An outbreak investigation done by Abraham *et al* in Tamil Nadu[22] has reported that water was the source of *Y. enterocolitica*, which had contaminated the butter milk. Sinha *et al* in their study at Delhi have isolated 11 strains of *Y. enterocolitica* from 50 sewage samples[8] Singh *et al* reported the presence of *Y. enterocolitica* in the throat swab of swine (32.9%)[18] and also in ground water (2.8%), waste water and Yamuna river water (9%) in Delhi. They have concluded that swine is the major source of infection and *Y. enterocolitica* is a water-borne pathogen [Table 7].

Table 7: Isolation rates comparison

Study	Stool samples		Environmental samples	
	Total number	Positive	Total number	Positive
Varghese *et al*	1470	03	Not done	
Ram *et al*	235	07	Not done	
Mathew *et al*	11	01	Bore well	01
Jones *et al*	09	02	Chitterlings	01
Okwori *et al*	150	06	Not done	
Lal *et al*	2000	41	Not done	
Singh *et al*	Not done		Pork	30
Sinha *et al*	Not done		Sewage (50)	11
Singh *et al*	1189	36	Swine (492)	162
			Ground (179)	05
			Waste water (73)	09
Present study	181	01	Environmental sample (15)	01

Isolation of *Y. enterocolitica* from the river water sample suggests that *Y. enterocolitica* is found in the environment in India and is a possible agent that causes outbreaks of gastroenteritis. This positive finding could also be due to contamination of river bank by excreta of swine or stools of patient with diarrhoea. A long-term study involving large number of samples will help in knowing its distribution in this region of India.

CONCLUSION

Y. enterocolitica is a zoonotic enteric pathogen commonly causing self-limiting intestinal infection.

This study showed that conventional direct plating method was not useful for *Y. enterocolitica* isolation. Use of 0.5% KOH as an enrichment broth and incubation at 4°C for 7-14 days will not only help in improved *Y. enterocolitica* isolation rate, but also suppress the growth of other enteric organisms.

During the course of the study, an innovative double enrichment technique using KOH for digestion followed by addition of PBS and refrigeration was attempted. But this method was not useful.

An incidental finding during this study was isolation of *Aeromonas* bacterial species from the river water and *E. coli* from environmental samples. This is a warning to the public health department to implement strict hygienic measures along the river bank because *Aeromonas* species are known agents causing outbreak of gastroenteritis, often ending fatally due to complications.

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