

Characterization of Pathogenic Strains of *Yersinia Enterocolitica* in and Around Chandigarh, India

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Abstract: The present work was carried out to study the occurrence of *Y. enterocolitica* in different samples in and around Chandigarh, India. 495 stool samples from diarrheal patients were collected for bacterial isolation. To identify the bacteria belonging to family Enterobacteriaceae, the samples were cultured on MA (MacConkey Agar), XLD (Xylose lysine deoxycholate) and SS (Salmonella shigella) agar. The bacteria were then subjected to biochemical tests, biotyping and serotyping to identify the pathogenic and non pathogenic strains of *Y. enterocolitica*. The pathogenicity of the isolated pathogenic strains was cross-verified by rabbit ileal loop test. In the present study, 5 out of 8 isolated strains from human stool samples were shown to harbor the pathogenic strains of bacterium. These pathogenic strains belonged to the biotype 1B and serotype 7, 8-8-13-8, 19. The non pathogenic strains belonged to the biotype 1A and serotype 41, 42-41, 43. Further, histopathological studies of infected intestines revealed destruction of villus architecture and mixed inflammatory cell infiltration. This is the first report of the prevalence of highly pathogenic strain of *Y. enterocolitica* 1B from stool samples in and around Chandigarh, India. This suggests that further studies are required to control the bacterial infection in this area.

Keywords: Bacteria, Biotype, Diarrhea, Serotype, *Yersinia enterocolitica*

I. Introduction

Yersinia enterocolitica, an important food and water-borne gastrointestinal agent is regarded as an emerging pathogen worldwide [1]. It causes acute gastroenteritis, enterocolitis and mesenteric lymphadenitis, as well as a variety of extra-intestinal problems. In several temperate or cold regions, *Y. enterocolitica* is frequently responsible for diarrheal diseases and its incidence almost rivals those of Salmonella and Campylobacter [2]. Diarrhea is the second leading killer of children, and nearly one in five children under the age of five dies as a result of dehydration, weakened immunity or malnutrition associated with diarrhea [3]. Diarrhea outbreaks are not still uncommon in India [4]. However, in the Indian scenario, the earliest reports about the isolation of *Y. enterocolitica* have been those of clinical samples. Subsequently, few studies have revealed the prevalence of *Y. enterocolitica* and other *Yersinia* spp. in samples of different food products including traditional fast foods [5]. Although there is a considerable current interest regarding *Y. enterocolitica* induced diarrhea throughout the globe only scanty literature is available about the same in the Indian context. Therefore the objective of this study was to assess the prevalence of potential pathogenic *Yersinia* spp. in stool samples through the use of biochemical and serological methods and also analyze them by protein characterization.

II. Materials And Methods

2.1. Samples

A total of 495 stool samples of diarrhea patients visiting Primary health centres and private registered medical practitioners in slums colonies in and around 5 kms of Chandigarh, India were collected. The patients on antibiotics were excluded from the study for taking stool samples. The stool samples were collected in Carry and Blair transport media and immediately transported to Department of experimental Medicine and Biotechnology, PGIMER, Chandigarh. In addition, the control strain of *Yersinia enterocolitica* (IP28205 & IP28206) and *Salmonella typhimurium* (ATCC13311) were maintained in the laboratory.

2.2. Ethical clearance

Administrative approval was taken from the authorities before commencement of research work. Medical superintendent, General hospital Sector-16, Chandigarh, India approved sample collection from primary health centers and dispensaries in and around Chandigarh. The animal ethical clearance for rabbit was obtained from the Institutional Animal ethics Committee, Panjab University, Chandigarh.

2.3. Isolation and Identification of *Yersinia* Species

For isolating pathogenic *Yersinia enterocolitica*, the stool samples were subjected to cold enrichment in PBS (1/15M, pH 7.6) for 21 days at 4°C [6]. The cold enriched samples were mixed with 0.5% KOH in 0.5%

NaCl for 10-15 sec followed by streaking on MacConkey agar (MA) and other enteric media (XLD and SS agar) and incubated for 24-48 hrs both at 37 °C and 22 °C [7].

The presumptive *Yersinia* isolates, which showed bull's eye colony morphology on CIN (cefsulodin-Irgasan-Novobiocin) media were cultured to get pure growth. All the isolates from pure culture were examined for Gram staining, catalase and oxidase tests, Motility tests and Christensens urea agar tests.

2.4. Confirmation of *Yersinia enterocolitica*

All the isolates which were gram negative, gave positive test for utilization of urease, negative for citrate and oxidase activity. These were submitted to further testing. For identification and biogrouping of isolates as *Y. enterocolitica*; 25 biochemical tests were performed.

2.5. Testing for pathogenicity markers

The invasiveness of *Y. enterocolitica* was tested by Congo red dye uptake as described by Statner and George [8].

2.6. Serotyping and Biotyping of isolated strains of *Yersinia enterocolitica*

Suspected pathogenic and non pathogenic strain of *Yersinia enterocolitica* from different samples were sent to the Pasteur Institute, Paris in wax sealed sterile containers containing 50% nutrient agar and 50% glycerol for confirmation and serotyping.

2.7. Outer Membrane Protein (OMP) Preparation

The isolated bacterial colonies were grown at 37 °C in MA agar. OMP preparation was done according to the method of Shin et al. [9].

2.8. Rabbit ileal loop test (RILT)

Overnight subcultures grown at 22°C and 37°C were diluted 1:20 in fresh LB and BHI and incubated for 2 hrs at 37°C. The bacteria were then centrifuged at 4000rpm, washed once and then resuspended in PBS(7.2) at a concentration of 10⁸ cfu/ml. The desired bacterial concentration was adjusted and checked by plating serial dilution of the sample on agar and counting cfu/ml after incubation at 25°C for 24-48hrs of growth. The live bacteria cfu/ml (LD 50) doses were checked for the presence of enteric toxicity by rabbit ileal loop test as described by De and Chaterjee [10].

2.9. Histopathological studies

Histopathological sections of control (PBS), bacterial toxin and endotoxin treated rabbit ileum were prepared by fixing in 10% formalin, stretched in hot water on albumin-coated slides, and stained with Delafield's haematoxylin/eosin technique to study histology [11].

III. Results

3.1. Colony identification and confirmation

All the 495 stool samples collected were cultured on MA, XLD and SS agar. 352 samples were found with colony characteristics for NLF (non lactose fermenting) family Enterobacteriaceae. These 352 colonies were further tested by gram staining. 285 colonies were observed to contain gram negative bacilli.

Since, *Yersinia* is gram negative, all these colonies were then subjected to catalase and oxidase test. All the gram negative bacteria showing positive catalase test and negative oxidase test were included in enterobacteriaceae family. These samples were further tested for urease and motility tests to identify *Yersinia enterocolitica*. In the motility test, 8 human stool samples showed motile bacteria at 22 °C. These bacteria lost their motility at 37 °C. Since this is the characteristic of *Yersinia*, these 8 human stool samples were labelled as *Yersinia enterocolitica* positive.

To further confirm the bacterial species, a number of biochemical tests were carried out. All the 8 samples produced urea, hydrolyzed ornithine and were unable to hydrolyze lysine. Also, these isolated strains were observed to be indole positive. However, the esculin hydrolysis capacities of strains were variable. From the 8 samples which were observed to contain *Yersinia enterocolitica*, 5 samples were unable to hydrolyze esculin. This is because these samples harboured the pathogenic strain of the bacterium and the rest 3 samples contained the non pathogenic strain of the bacterium. These results were proved by Congo red dye uptake tests. Pathogenic bacteria gave positive test and non- pathogenic bacteria gave negative test (Table 1).

3.2. Biotyping and Serotyping

These isolated strains were sent to Pasteur institute, Paris; the referral centre for *Yersinia* for confirmation. All the five pathogenic strains were reported to be in biotype 1B & serotype 7,8-8-13-8, 19 (CNR

No- IP28202, IP28201, IP28201, IP28204 and IP28205). The non pathogenic strain were classified into biotype 1A& serotypes 41,42-41,43 (CNR No- IP28206).

3.3. Protein Characterization

All the isolated strains of *Yersinia* showed the presence of a 38kDa OMP protein irrespective of pathogenicity. However, all the pathogenic strains also showed 17kDa OMP which was absent in the non-pathogenic strains (Fig 1).

3.4. Rabbit ileal loop test and histopathological studies

It was observed that intestinal loop injected with the isolated virulent strain dilated due to the accumulation of water proving the presence of the bacterial strain that causes secretory diarrhea. The same was observed with the control virulent strain but not with the control loop injected with the buffer.

Further the sections of the intestine exposed to virulent strains showed destruction of villous architecture with loss of goblet cells and mixed inflammatory cell infiltration (neutrophils and lymphocytes) when compared with the sections of control loops injected with buffer. The villous architecture altered, the sub-mucosa was disorganized, mucosal hemorrhage and denudation of epithelial inflammation and edema was observed in the intestine (Fig 2, Fig 3).

IV. Discussion

Yersinia enterocolitica is a versatile enteropathogen that, most commonly, causes gastroenteritis in humans [12]. Since its recognition as a distinct species, it has been isolated from different materials such as human stools, intestinal contents of pigs, dogs and rodents and foodstuffs such as pork, beef, chicken, milk, vegetables and drinking water. In spite of all these isolations, the epidemiology of *Y. enterocolitica* infections is complex and remains poorly understood [13]. To add to it, very little is known about this pathogen in the Indian context. Therefore, in the present study, the prevalence of *Yersinia enterocolitica* in stool samples of diarrheal patients was worked out. In this study, out of 495 diarrhea stool samples worked with, 8 stool samples showed presence of *Yersinia enterocolitica*. Out of these, 5 samples proved to be pathogenic by Congo Red Dye Uptake Test and classified as highly pathogenic biotype 1B by WHO reference center for *Yersinia* spp., Pasteur Institute, Paris. To the best of our knowledge this is for the first time that the highly pathogenic biotype of *Yersinia enterocolitica* is being reported from stool samples of patient suffering from diarrhea in India. In all the isolated 8 strains of *Yersinia enterocolitica*, a 38 kDa outer membrane protein was observed to be present. This finding corroborates with the fact observed by the earlier researchers which clearly shows the presence of 38 kDa outer membrane protein in all strains of *Yersinia enterocolitica* regardless of its virulence [9]. Apart from this, 17kDa OMP was found to be observed in only the pathogenic strain. This particular observation also correlates with the known fact that the expression product of pathogenic ail gene is a 17kDa protein, which mediates bacterial attachment to some cultures epithelial cell lines and invasion of others [14].

We believe that this data is only the tip of the iceberg and wide scale epidemiological survey should be done to evaluate the incidence and prevalence of the highly pathogenic strain in Indian context and to formulate therapeutic/ diagnostic/ prevention strategies to combat human pathogenesis due to the organism; failing which we will not be appropriately prepared to tackle community infection caused by the pathogen in situation of epidemics/endemics in decades to come.

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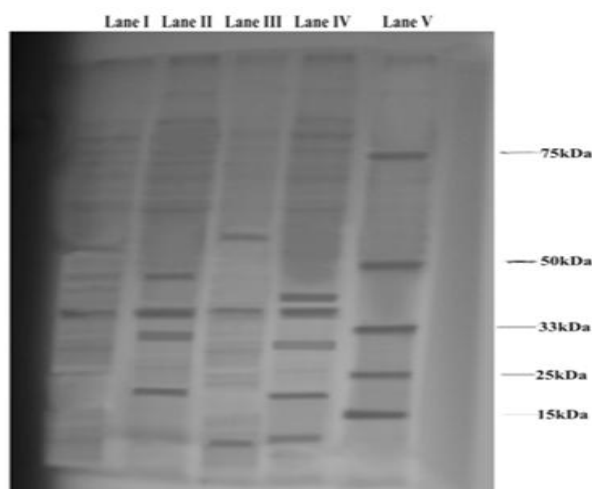


Fig (1) SDS-PAGE gel picture of purified outer membrane proteins (OMPs) of *Yersinia enterocolitica* isolated from stool of four diarrhea patients. Lane V shows the bands of molecular weight markers. Lane IV & II shows the characteristic bands of 17kDa OMP isolated from two pathogenic strains. Lane I & III show the OMP pattern of nonpathogenic *Yersinia enterocolitica* isolated from stool samples

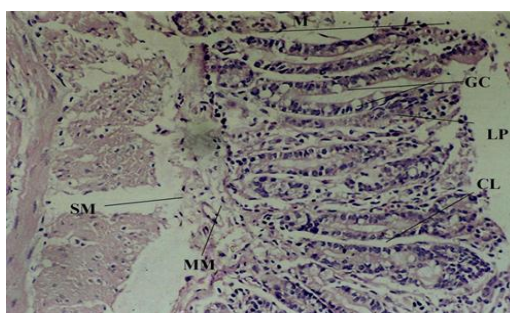


Fig (2) Histopathology of normal intestinal epithelium (20X). LA-lamina propria, GC-goblet cells, CL- crypt of lieberkhun, MM-muscularis mucosa, SM-submucosa and M-Mucosa

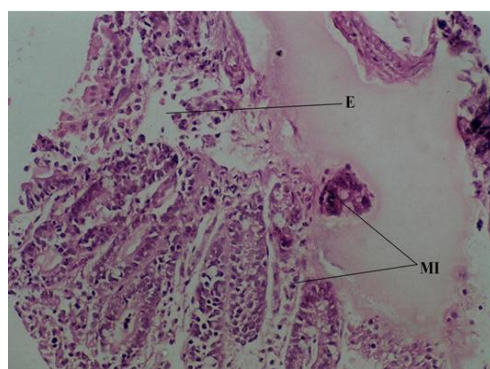


Fig (3) Histology of intestinal epithelium (20X) of rabbit ileal loop challenged with virulent strain of *Yersinia enterocolitica*. E- Edema, MI- Mixed Inflammation

Name of the test	°Ref1	°Cont-1	°Cont-2	Results of isolates from eight stool samples								°Ref2	°Cont-3	
				1	2	3	4	5	6	7	8			
ONPG	+	+	+	+	+	+	+	+	+	+	+	+	-	-
LYSINE	-	-	-	-	-	-	-	-	-	-	-	-	+	+
ORNITHIN	+	+	+	+	+	+	+	+	+	+	+	+	+	+
UREASE	V	+	+	+	+	+	+	+	+	+	+	+	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NITRATE-REDUCTION	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H ₂ S REDUCTION	-	-	-	-	-	-	-	-	-	-	-	-	+	+
CITRATE-UTILIZATION	-	-	-	-	-	-	-	-	-	-	-	-	+	+
VOGES-PROSKAUERS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
METHYL RED	+	+	+	+	+	+	+	+	+	+	+	+	+	+
INDOLE	V	+	+	+	+	+	+	+	+	+	+	+	-	-
MALONATE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ESCULIN HYDROLYSIS	V	-	+	-	+	-	-	+	-	-	+	-	-	-
ARABINOSE	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XYLOSE	V	+	+	+	+	+	+	+	+	+	+	+	+	+
ADONITOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RHAMNOS	-	-	-	-	-	-	-	-	-	-	-	-	+	+
CELLIBIOSE	V	-	-	-	-	-	-	-	-	-	-	-	-	-
MELIBIOSE	-	-	-	-	-	-	-	-	-	-	-	-	+	+
SACCHAROSE	+	+	+	+	+	+	+	+	+	+	+	+	-	-
RAFFINOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TREHALOSE	+	+	+	+	+	+	+	+	+	+	+	+	-	-
GLUCOSE	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LACTOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OXIDASE	-	-	-	-	-	-	-	-	-	-	-	-	Nil	Nil

Table 1: Biochemical test results of 8 isolated *Yersinia enterocolitica* strains from the along with the control strains.

a and b- two control strains of *Yersinia enterocolitica*, d- *Salmonella typhimurium* control strain, c and e- The reference test result with *Yersinia enterocolitica* and *Salmonella typhimurium* as indicated in kit, + positive biochemical test, - indicates negative biochemical test.