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Isolation, biochemical characterization and *in vitro* tests of pathogenicity of *Yersinia enterocolitica* isolated from pork

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 diarrhoeal diseases and its incidence almost rivals those of *Salmonella* and *Campylobacter*[2]. Recently, a food-borne outbreak of gastroenteritis involving 25 persons in North Arcot district of Tamil Nadu was attributed to *Y. enterocolitica*[3]. In India, *Y. enterocolitica* has earlier been isolated from stools of diarrhoeic patients[4–7], milk[8], pig intestinal contents[4] and rectal swabs[9]. Recently, in our laboratory, *Y. enterocolitica* was isolated from sewage effluents in Delhi (unpublished). Although pigs represent the single most important reservoir of this organism and pork has been implicated in a number of outbreaks in several parts of the world[10], there has been no report on the isolation of *Y. enterocolitica* from pork in India. The present study reports isolation, biochemical characterization and *in vitro* tests of pathogenicity of *Y. enterocolitica* from pork collected from a local slaughterhouse.

Twelve samples of pork, each from a separate animal, were collected over a period of two weeks from a local slaughterhouse-cum-piggery farm. Samples were collected in sterile petri plates from inside the thigh muscle to avoid surface contamination. These were transported to the laboratory within 20 min and refrigerated immediately. All pork samples were processed for isolation of *Y. enterocolitica* within 1 h of collection. 25 g of sample was homogenized in a blender and added to 225 ml of phosphate-buffered saline containing sorbitol (1%) and bile salts (1.5%), and kept at 4°C up to 3 weeks. After 2 and 3 weeks, the cold-enriched samples were subjected to alkali treatment by adding 0.5 ml of 0.5% KOH into 4.5 ml of cold-enriched samples for 5, 15 and 30 s (ref. 11). The cold-enriched and alkali-treated, and also non-alkali-treated samples were streaked on to cefsulodin–irgasan–novobiocin (CIN) and MacConkey agars (Hi Media) and incubated at 25°C. After 18–24 h of incubation, presumptive *Y. enterocolitica* colonies were identified by studying these both by the naked eye as well as under a stereomicroscope (10 ×) (Leica). The colonies showing characteristic bulls-eye morphology with deep red center and clear colourless periphery on CIN agar and non-lactose-fermenting (NLF) on MacConkey agar were selected for detailed biochemical characterization. Suspected *Y. enterocolitica* isolates were maintained as both slant and stab cultures in nutrient agar and stored at 4°C.

Colonies conforming to the above cultural criteria were initially subjected to four biochemical tests, viz. urease, Kligler's iron agar (KIA), differential motility and Voges-Proskauer (VP). Based on these results, the suspected isolates were subjected to detailed biochemical characterization[12]. Species identification was done by fermentation of rhamnose, melibiose, α-methyl-D-glucoside, sucrose and raffinose. Isolates identified as *Y. enterocolitica* were biotyped according to Wauter's biotyping scheme[13]. Samples were sent to Institut Pasteur, Paris for confirmation and serotyping.

In vitro tests for pathogenicity, viz. pyrazinamidase activity, CR–MOX (Congo red–magnesium oxalate) agar, autoagglutination, salicin, esculin, D-xylose fermentation and crystal violet binding were carried out as recommended[14,15]. A virulence plasmid-bearing *Y. enterocolitica* reference strain (W22703, serotype O:9) was used concurrently as control for these tests.

Thirty isolates were picked up from CIN agar and MacConkey agar plates based on their colony morphology. Cold enrichment of the samples for 3 weeks was found to be better in yielding colonies of suspected *Y. enterocolitica* (24 isolates) compared to enrichment for 2 weeks (6 isolates). Also most of these isolates were obtained after the cold-enriched samples were treated with alkali for 15–30 s. Lack of alkali treatment or treatment of samples for 5 s was unsatisfactory as the presence of the background micro-flora marred the isolation of *Y. enterocolitica*.

The results of biochemical characterization of the isolates are summarized in Table 1. Biochemical tests identified 14 of the isolates from six pork samples as *Y. enterocolitica* while two isolates from two samples were found to be *Morganella morganii* subsp. *morganii* (biotype C). All isolates of *Y. enterocolitica* belonged to biotype 1A. Six

Table 1. Biochemical characteristics of *Y. enterocolitica* and *M. morganii* isolates from pork

Characteristics	*Y. enterocolitica* (n = 14)	*M. morganii* (n = 2)
Urease (Christensen's)	+	+
Kligler's iron agar	K/A	K/A
Motility 25°C/37°C	+/–	+/–
Voges–Proskauer 25°C/37°C	+/–	–/–
Lysine decarboxylase (Moller)	–	–
Arginine decarboxylase (Moller)	–	–
Ornithine decarboxylase (Moller)	+	–
Phenylalanine deaminase	–	–
Citrate utilization (Simmons')*	–	–
Methyl red	+	–
Catalase	+	+
Oxidase	–	–
Indole production	+	–
Nitrate reduction	+	–
Growth in KCN	–	+
Malonate utilization	–	–
O-nitrophenyl-β-D-galactopyranoside	+	–
Gluconate	–	–
Glucose, galactose, mannose	A/–	A/–
Cellobiose, fructose, inositol, maltose, mannitol, salicin, sorbitol, trehalose, D-xylose, sucrose	A/–	–/–
D-arabinose, adonitol, dulcitol, inulin, lactose*	–/–	–/–
Rhamnose, melibiose, α-methyl-D-glucoside, raffinose	–/–	–/–

All incubations were carried out in triplicate at 25°C for 3 days unless indicated otherwise; *incubated for 7 days; K/A, alkaline slant/acid butt; A/–, acid/no gas; –/–, no acid/no gas.

Table 2. *In vitro* tests of pathogenicity for *Y. enterocolitica* isolates (n = 6) from pork

Test	Result
Congo red–magnesium oxalate (CR–MOX) agar	+
Crystal violet binding	+
Autoagglutination	–
Pyrazinamidase activity	+
Salicin fermentation	+
Esculin hydrolysis	–
D-xylose utilization	+

representative isolates sent to *Yersinia* National Reference Laboratory and WHO Collaborating Center, Institut Pasteur, Paris for confirmation and serotyping, have been given Institut Pasteur Collection Numbers as IP 26148, IP 26149, IP 26150, IP 26151, IP 26152 and IP 26153. Four were serotyped as O:7,8-8-8,19 and two were non-typable. Three cultures have been deposited with MTCC, IMTech., Chandigarh (MTCC 3234, MTCC 3237, MTCC 3238).

The results of the *in vitro* tests to determine the pathogenicity of six isolates of *Y. enterocolitica* each representing a separate pork sample are shown in Table 2. All the isolates were positive for CR–MOX test as evidenced by the appearance of small pin-point red colonies after 48 h of incubation at 37°C. All the isolates were also positive for crystal violet binding and pyrazinamidase tests.

Pigs are the most important reservoir of *Y. enterocolitica*[10]. In India, *Y. enterocolitica* has been isolated from the intestinal contents of a slaughtered pig[4], and rectal swabs of apparently healthy pigs[9]. Serological evidence of its existence in these animals has also been presented[16]. Isolation of *Y. enterocolitica* from pork is yet another evidence of the presence of this organism in India. The serotype O:7, 8-8-8, 19 has not been isolated in any of the earlier studies in India. Those reported from intestinal contents and rectal swabs of pigs belonged to O:9, 16, and O:3 and O:9, respectively. The better recovery of *Y. enterocolitica* only after three weeks of cold enrichment indicated lower count in pork.

All the isolates recovered in this study belonged to biotype 1A. Though regarded as non-pathogenic in the past[10], there has been a renewed interest in the pathogenicity of such isolates. Evidences indicate that *Y. enterocolitica* biotype 1A may cause self-limiting enteritis without associated systemic symptoms or even gastroenteritis similar to those of virulent biotypes[17]. Incidentally, in several parts of the world a significant proportion of *Y. enterocolitica* isolated from clinical cases of gastroenteritis has been found to be of biotype 1A[18]. Recently, it has been suggested that biotype 1A *Y. enterocolitica* may be pathogenic by some novel, as yet undetermined mechanisms[19].

Among the various *in vitro* tests of pathogenicity, CR–MOX positivity, i.e. calcium-dependent growth and Congo red binding, which are plasmid and chromosomally-mediated markers, respectively, indicated pathogenic potential of these isolates. So was the crystal violet binding and esculin negativity. Burnens *et al.*[17] found the CR–MOX test to be most reliable for determining pathogenicity. However, the results of other *in vitro* tests of pathogenicity, viz. pyrazinamidase, autoagglutination, and salicin and D-xylose fermentation denote the non-pathogenicity of these isolates. As no individual *in vitro* virulence marker is a single reliable indicator of virulence, the pathogenicity of these isolates remains inconclusive. Further, *in vivo* tests using animals and detection of molecular markers of pathogenicity based on virulence plas-

mid-derived DNA probes or *yst* (*Yersinia* stable toxin) locus would help in confirming the pathogenic potential of these isolates. The worldwide isolation of biotype 1A *Y. enterocolitica* from human, animals and diverse environments, as well as renewed interest in their pathogenicity, warrant further studies.

M. morganii, regarded as an opportunistic pathogen, is mainly implicated in the urinary tract and wound infections in debilitated post-surgical patients. Among a few reports of its isolation from India, it has been reported from diarrhoeic patients[20], urinary tract stones[21] and even vegetable salads[22]. *M. morganii* subsp. *morganii* (biotype C) are rare[23]. Some of the biochemical characteristics of these isolates, especially indole and methyl red negativity, which have earlier been reported to be positive, were noteworthy. The significance of isolation of *M. morganii* from pork needs to be determined further.

This is the first report of isolation of *Y. enterocolitica* from pork in India. The present study has revealed that it would be worthwhile to further look for *Y. enterocolitica* in pigs slaughtered for pork so that the magnitude of its prevalence may be ascertained.

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Cyanobacteria from extreme acidic environments

Cyanobacteria (CB) or blue-green algae are capable of both carbon assimilation and N_2-fixation, thereby enhancing productivity in a variety of environments[1]. Among soil properties, pH is a very important factor in growth, establishment and diversity of CB which have generally been reported to prefer neutral to slightly alkaline pH for optimum growth[2,3]. Acidic soils are therefore one of the stressed environments for these organisms and these are normally absent at pH values below 4 or 5: eukaryotic algae, however, flourish under these conditions[4,5]. There are no reports on the existence of CB below this pH level. The present communication however reports on the occurrence of a wide variety of CB in the extremely acidic soils of Kerala (below pH 4).

Composite samples comprising 10 subsamples each were collected along a transect in the peaty bog lands and adjoining wet paddy fields, potentially acidic areas of Alappuzha district, Kerala. Acid sulphate soils in India are confined to this area and form saline peaty soils locally known as *kari* soils. The development of soil acidity is generally believed to be associated with the base unsaturation caused by leaching out of bases and genesis from base-poor acidic rocks[6]. The dissolved or free acidic substances, such as sulphuric acid and ferric and aluminium sulphate, accentuate acidity in acid sulphate soils[7]. Soil samples were wet or in almost suspension form; pH of the samples was determined using a Systronics