INTRODUCTION

Yersinia enterocolitica is a versatile enteropathogen that, most commonly, causes gastroenteritis in humans (Bottone, 1999). The heterogeneous nature of Y. enterocolitica has led to its division into six biotypes, namely 1A, 1B, 2, 3, 4 and 5. The virulence of primary pathogenic biotypes, i.e. biotypes 1B and 2–5, is attributed to the presence of a virulence plasmid termed pYV, chromosomal invasion-associated genes, namely ail and inv, and enterotoxin gene ystA (Cornelis et al., 1987; Miller & Falkow, 1988; Delor & Cornelis, 1992). Biotype 1A strains, however, have been reported to lack such virulence markers (Miller et al., 1989; Pierson & Falkow, 1990; Delor & Cornelis, 1992). Consequently, in the past, these strains were regarded as avirulent. Nevertheless, biotype 1A strains have been associated with nosocomial and food-borne outbreaks of diarrhea (Ratnam et al., 1982; McIntyre & Nnochiri, 1986; Greenwood & Hooper, 1990; Butt et al., 1991). Studies have also indicated that gastroenteritis caused by biotype 1A strains is indistinguishable from that caused by primary pathogenic biotypes (Morris et al., 1991; Burnens et al., 1996). Recently, Grant et al. (1998, 1999) showed that some of the biotype 1A strains of Y. enterocolitica invaded epithelial cells and resisted killing by macrophages, though to a lesser extent than the primary pathogenic strains.

Primary pathogenic strains of Y. enterocolitica are known to produce a heat-stable enterotoxin named Yersinia stable toxin-a (YST-a) (Delor et al., 1990). Studies with YST-a-negative mutants and their transcriptional analysis suggested that YST-a contributes towards virulence of Y. enterocolitica (Delor & Cornelis, 1992; Mikulskis et al., 1994). However, the role of other variants of YST, i.e. YST-b and YST-c, in the pathogenicity of Y. enterocolitica, especially biotype 1A strains, is not well understood. This is mainly because these toxins are known to be produced only in vitro at temperatures below 30 °C. Also, the presence of a yst gene does not necessarily correlate with production of toxin in vitro (Ramamurthy et al., 1997; Grant et al., 1998). In a recent study conducted in Delhi (India), we isolated Y. enterocolitica from diverse sources, viz. stools of diarrhoeic patients, swine throats, river water, wastewater and groundwater (Singh et al., 2003). All isolates belonged to biotype 1A. The aim of the present study was to investigate these strains for enterotoxigenicity per se and the presence of Yersinia stable toxin genes (YST-A, YST-B and YST-C).

METHODS

Bacterial strains. Two hundred and sixteen strains of Y. enterocolitica biotype 1A comprising 36 strains isolated from diarrhoeic stools of paediatric patients, 162 from swine throats and 18 from various aquatic sources (river, wastewater and groundwater) were used in this study. In addition, 27 isolates of Yersinia intermedia (4 from diarrhoeic stools and...
23 from swine) and 16 strains of *Yersinia frederiksenii* (7 from diarrhoeic patients and 9 from swine) were also used. Details of these strains have been reported previously (Singh et al., 2003). Virulence-plasmid-bearing strains of *Y. enterocolitica*, viz. IP134, IP26332, IP885, IP383 and IP26329, procured from the *Yersinia* National Reference Laboratory and WHO Collaborating Center, Institut Pasteur (Paris), were used as standards. *Escherichia coli* HB101 was used as the negative control.

**Production of YST in vitro.** For enterotoxin production, bacteria were grown in tryptic soy broth (TSB) containing 0.6 % (w/v) yeast extract. The inoculated medium was incubated with shaking (250 r.p.m.) at 28 °C for 48 h, and at 37 °C for 48 h and 144 h. In order to study the production of enterotoxin at an alkaline pH of 7.5 at 37 °C (the conditions found in the ileum), the test strains were inoculated in brain heart infusion (BHI) broth supplemented with 5 mM CaCl₂, 0.1 M MOPS and 0.1 M NaCl (Mikulskis et al., 1994). The cultures were grown at 37 °C for 48 h under shaking conditions (250 r.p.m.). The culture supernatant was obtained by centrifugation at 18 000 r.p.m. for 15 min at 4 °C and sterilized by membrane filtration (0.2 μm; Millipore). The filtrate was heated to 100 °C for 10 min and aliquots were stored at −40 °C.

**Assay for YST activity.** Enterotoxin activity was assessed in infant mice as described by Dean et al. (1972). Groups of two to five Swiss mice, 2–4 days old, were inoculated intragastrically with 100 μl of the test filtrate in which 0.01 % Evans blue dye was incorporated as a marker. After 3 h, mice were sacrificed by inhalation of chloroform. The ratio of the intestinal weight to the remaining body weight was determined. A ratio greater than 0.09 was considered indicative of enterotoxin production (Dean et al., 1972). Five virulence-plasmid-bearing strains of *Y. enterocolitica* (IP134 and IP26332: serotype O:3, biotype 4; IP885 and IP26329: serotype O:9, biotype 2; IP885: serotype O:5,27, biotype 2) were used as positive controls. For the positive controls, the intestine to body weight ratio was 0.092 for the individual mice. Saline and growth medium (TSB and BHI), as well as *E. coli* HB101 was used as the negative control. The comparable values for intestine to body weight ratios were always below 0.065. All assays were done in a group of a minimum of three mice each.

**Detection of Yersinia stable toxin (yst) genes.** All *Yersinia* isolates were studied for the presence of yst genes, viz. ystA, ystB and ystC, by dot blot hybridization using specific oligonucleotide DNA probes. Standard techniques with some modifications were used for dot blot hybridization (Sambrook & Russel, 2001). Oligonucleotides were custom-made by Genset Oligos (Singapore). The oligonucleotide probe sequence for ystA was 5′-GCTTTGTATCCTCGTGTCGCAC-3′, corresponding to nucleotides 243–264 of the ystA sequence (Delor et al., 1990); the oligonucleotide probe sequence for ystB was 5′-ACTCAGACCC-CATGCGCTCTGAGA-3′, derived from nucleotides 232–255 of the ystB gene (GenBank accession no. D88145); and the sequence of the probe for ystC was 5′-GTTGTTGATGTATCATGCACAACTAG-3′, corresponding to nucleotides 79–108 of the ystC gene (Huang et al., 1997). The oligonucleotides were end-labelled with [γ-32P]dATP (Board of Radiation and Isotope Technology, Hyderabad, India) using T4 polynucleotide kinase (MBI Fermentas). The DNA probes were purified by column chromatography using Sephadex G-50 (Sambrook & Russel, 2001). Hybridization was performed for 21 h at 57 °C (for ystA and ystB) and 56 °C (for ystC) in a solution containing 3× SSC (1× SSC contains 0.15 M NaCl and 15 mM sodium citrate), 0-2 % SDS, 2× Denhardt’s solution and 200 μg denaturated sonicated salmon sperm DNA ml⁻¹ along with the respective probe. Membranes were finally washed at high stringency with 0.1 % SSC at the respective hybridization temperatures for 15 min, and exposed for 24 h to X-ray film (Kodak) at −70 °C for autoradiography.

**RESULTS AND DISCUSSION**

Production of enterotoxin is an important virulence attribute of most enteropathogens. To determine the entero-toxigenicity, all isolates were examined for production of YST *in vitro*. Under the conditions that are generally used for the production of enterotoxin by *Y. enterocolitica*, i.e. production at 28 °C for 48 h in TSB supplemented with yeast extract, 77.7 % of clinical and 62.3 % of swine isolates produced enterotoxin (Table 1). However, in an earlier study on *Y. enterocolitica* biotype 1A isolates collected from diverse geographic regions of the world, Grant et al. (1998) reported that 25.3 and 40.6 % of strains of clinical and non-clinical origin, respectively, produced enterotoxin. Earlier, Pai et al. (1978) showed that production of enterotoxin by biotype 1A strains was lower (39 %) compared to primary pathogenic strains (99 %). The higher proportion of enterotoxin-producing strains in the present study may be attributed to differences in strains present in different parts of the world. Although *Y. enterocolitica* biotype 1A strains have been shown to produce heat-stable enterotoxin (YST-b) at 28 °C, its role in pathogenicity remains unproven (Grant et al., 1998). This is because of the absence of reports showing the production of YST-b at 37 °C, the temperature of the

---

**Table 1. Production of YST *in vitro* by *Y. enterocolitica* isolates under various culture conditions**

Enterotoxin production was determined by infant mouse assay; the bacteria were grown in TSB. None of the 18 isolates from water produced enterotoxin.

<table>
<thead>
<tr>
<th>Source (no. of isolates)</th>
<th>No. of strains producing enterotoxin (%) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 °C (48 h)</td>
</tr>
<tr>
<td>Clinical (36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 (77-7)</td>
</tr>
<tr>
<td>Swine (162)</td>
<td>101 (62-3)</td>
</tr>
</tbody>
</table>

*Grown in BHI broth at pH 7.5 for 48 h.*
Yersinia enterocolitica biotype 1A enterotoxin

body of the host. Although YST-a is also produced at temperatures below 30 °C in a normal medium (Amirmozafari & Robertson, 1993), Mikulskis et al. (1994) showed that ystA transcription can be induced at 37 °C by maintaining the pH of culture medium at that present in the lumen of the ileum, i.e. pH 7.5. To determine the role of YST-b in the pathogenicity of Y. enterocolitica biotype 1A, all isolates were studied for its production at 37 °C. Under normal culture conditions (i.e. when the pH of the culture medium was allowed to change with the growth of the bacteria) in TSB, most Y. enterocolitica isolates failed to produce enterotoxin at 37 °C after 48 h incubation (Table 1). Only two isolates of Y. enterocolitica biotype 1A produced enterotoxin under these conditions. On prolonged incubation (144 h) in TSB at 37 °C, however, the number of Y. enterocolitica isolates that produced enterotoxin increased to 19. To determine enterotoxin production at the pH found in the ileum, Y. enterocolitica isolates were grown at 37 °C for 48 h at a constant pH of 7.5. Under these conditions the proportion of the strains producing enterotoxin increased further and all Y. enterocolitica isolates that showed positive enterotoxin activity at 28 °C produced enterotoxin at 37 °C also (Table 1). These studies demonstrated the production of YST-b by Y. enterocolitica biotype 1A at 37 °C, and suggest that it may be produced in vivo in the gut also.

To determine the distribution of yst genes in Yersinia isolates, all the 259 isolates were subjected to hybridization with probes for ystA, ystB and ystC genes under highly stringent conditions and the results are summarized in Table 2. All the clinical and 96.3 % of the swine isolates of Y. enterocolitica biotype 1A hybridized with the probe for the ystB gene. None of the Y. enterocolitica strains hybridized with the probes for the ystA or ystC genes. Further analysis of the data revealed that all strains that produced enterotoxin in vitro were positive for the ystB gene. This indicated that ystB was the most prevalent and major contributor to enterotoxicity of Y. enterocolitica biotype 1A. All Y. enterocolitica isolates that produced enterotoxin in vitro were positive for the ystB gene. None of the strains showed hybridization with more than one yst probe. A number of Y. enterocolitica isolates that hybridized with the probe for ystB nevertheless failed to produce enterotoxin, suggesting the presence of a silent ystB gene. This phenomenon has been reported to be observed commonly for ystA (Nakao et al., 1995) and may be attributed to spontaneous mutations. Several workers in the past reported the absence or low prevalence of ystA in Y. enterocolitica biotype 1A strains (Delor et al., 1990; Ramamurthy et al., 1997; Durisin et al., 1998; Grant et al., 1998). Ramamurthy et al. (1997) in a study involving a total of 304 Y. enterocolitica isolates belonging to various biotypes reported that all the 36 ystB gene positive strains belonged to biotype 1A, and that 32 (89 %) of these produced enterotoxin. Grant et al. (1998) found that 87.3 and 81.3 % of clinical and non-clinical Y. enterocolitica biotype 1A strains, respectively, hybridized with a ystB probe. One of the strains showed hybridization with a probe for ystA, while none was positive for ystC. Moreover these workers also reported that most enterotoxin-producing strains hybridized with a ystB probe. Recently, Thoerner et al. (2003) also observed that of the 50 strains of biotype 1A, 43 (80 %) showed the presence of ystB, while only two isolates were positive for ystA. None of the studies have reported the ystB gene in strains other than biotype 1A (Ramamurthy et al., 1997; Grant et al., 1998; Thoerner et al., 2003). Thus it may be inferred that ystB is confined to Y. enterocolitica strains belonging to biotype 1A. Y. intermedia and Y. frederiksenii are known to lack classical Yersinia virulence markers and are thus generally considered as avirulent. In the present study too, none of these isolates produced enterotoxin. However, a hybridization signal with the probe for the ystA gene was observed in seven Y. intermedia strains while two Y. intermedia and three Y. frederiksenii isolates showed hybridization with the probe for ystB (Table 2). These results were similar to those in earlier studies by other investigators where Y. intermedia and Y. frederiksenii have been reported to lack enterotoxin production though some of the Y. intermedia strains did show a hybridization signal for the ystA gene (Kwaga et al., 1992; Sulakvelidze et al., 1996). However, Ramamurthy et al. (1997) reported that a strain of Y. intermedia isolated from the USA hybridized with ystB and secreted enterotoxin too. In the present study, the hybridization observed with probes for ystA and ystB genes may be due to either an inactive yst gene or the presence of a non-specific homologous region.

In conclusion, the present study showed that the ystB gene is widely distributed in biotype 1A strains of Y. enterocolitica and production of YST-b can be induced at the pH conditions found in the ileum, suggesting YST-b to be an important virulence determinant of Y. enterocolitica biotype 1A strains.

Table 2. Presence of ystA, ystB and ystC genes in Yersinia isolates

Determined by hybridization with the respective DNA probes; numbers in parentheses indicate percentage. No strains were positive for the ystC gene.

<table>
<thead>
<tr>
<th>Species (no. of isolates)</th>
<th>No. of strains positive for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ystA</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td></td>
</tr>
<tr>
<td>Clinical (36)</td>
<td>0</td>
</tr>
<tr>
<td>Swine (162)</td>
<td>0</td>
</tr>
<tr>
<td>Water (18)</td>
<td>0</td>
</tr>
<tr>
<td>Y. intermedia (27)</td>
<td>7 (25.9)</td>
</tr>
<tr>
<td>Y. frederiksenii (16)</td>
<td>0</td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENTS

We are grateful to Dr P. K. Burma, Dept of Genetics, UDSC, New Delhi, for his help in hybridization studies. We also thank Dr E. Carniel, Director, Yersinia National Reference Laboratory and WHO Collabor-
at Center, Institut Pasteur (Paris), for providing the standard strains of Y. enterocolitica. The study was supported by ICMR-SRF to I. S. and a research grant of DST to J. S. V.

REFERENCES


Int J Med Microbiol Downloaded from www.microbiologyresearch.org by


