Importance of *Providencia* species as a major cause of travellers' diarrhoea

Myonsun Yoh, Junko Matsuyama, Motoki Ohnishi, Kazuhiro Takagi, Hirozane Miyagi, Kazuhiro Mori, Kwon-Sam Park, Takahiro Ono and Takeshi Honda

1,2 Center for Emerging Infectious Diseases 1 and Department of Bacterial Infections 2, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

3 Kansai Airport Quarantine Station, 1 Senshu Kuko Naka, Tajiri-cho, Sennan-gun 549-0011, Japan

In this study the importance of *Providencia* species as a cause of travellers' diarrhoea was examined using a selective medium developed by the authors. *Providencia* species could easily be distinguished from other enteric pathogens by the colour of the colonies obtained. Nine strains of *Providencia alcalifaciens*, nine of *Providencia rettgeri* and five of *Providencia stuartii* were isolated from 130 specimens, representing a surprisingly high incidence of infection compared with other pathogens isolated on SS agar and TCBS agar. Patients infected with *P. rettgeri* complained of abdominal pain, as for other *Providencia* species, but also of vomiting, which is rather characteristic of *P. rettgeri* infection. To analyse the pathogenicity of these isolates, their invasiveness was examined using Caco-2 cells. Most of the *P. rettgeri* strains invaded Caco-2 cells. Random amplified polymorphic DNA (RAPD) fingerprinting showed the same profile for two *P. rettgeri* isolates from individuals travelling in the same tour group. The results show that *Providencia* species, especially *P. rettgeri*, might cause diarrhoea, and that these species are important pathogens.

**INTRODUCTION**

*Providencia alcalifaciens* has been described as a possible diarrhoea-causing pathogen in travellers and children in developing countries (Albert et al., 1998; Guth & Perrella, 1996; Haynes & Hawkey, 1989; Janda et al., 1998). In 1996, a large outbreak of food-borne infection, involving 290 patients, occurred in Japan. We investigated the aetiology of the large outbreak and concluded that the cause was *Providencia* species, especially *P. alcalifaciens* (Murata et al., 2001). To show the role of *Providencia* species in bacterial gastroenteritis, Janda et al. (1998) evaluated their ability to invade eukaryotic cell lines. They reported that some *P. alcalifaciens* strains recovered from people with diarrhoea were invasive in HEp-2 and other eukaryotic cell lines but *Providencia stuartii* and *Providencia rettgeri* were not. As the enteropathogenicity of the other species of *Providencia* has not been established, *Providencia* species other than *P. alcalifaciens* are considered to be commensal flora in the gastrointestinal tract, and their detection as pathogens in patients with diarrhoea is not usually attempted. Furthermore, selective media to isolate *Providencia* species have not been developed.

To evaluate not only *P. alcalifaciens* but also other *Providencia* species for their ability to cause diarrhoea, we established an effective selective medium (polymyxin-mannitol-xylitol medium for *Providencia*, PMXMP). A medium for the detection and recognition of *P. alcalifaciens* in faeces (*P. alcalifaciens* medium (PAM)) was previously reported (Senior, 1997). We modified PAM to detect *Providencia* species with ease and simplicity.

In the present study, we found a high incidence of *Providencia* species, especially *P. rettgeri*, as a cause of travellers’ diarrhoea, and examined the invasiveness of these pathogens in Caco-2 cells. Albert et al. (1998) reported vomiting in diarrhoeal children infected with *P. alcalifaciens*, but to our knowledge there have been no reports so far on adult patients infected with other *Providencia* species. The results of this study provide evidence that *P. rettgeri* strains can cause diarrhoea. This is the first report on the importance of *P. rettgeri* as a cause of gastroenteritis.

**METHODS**

**Bacterial strains.** *P. alcalifaciens*, *P. rettgeri*, and *P. stuartii* strains were isolated at the Kansai airport quarantine station in 2002 (Table 1). *P. alcalifaciens* RIMD 1656012, *P. stuartii* RIMD 1660001, *P. rettgeri* RIMD 1658001, *Providencia rustigianii* RIMD 1659002, *Escherichia coli* RIMD 0509770 (enterotoxigenic *E. coli*, ETEC), *E. coli* RIMD 0509952

**Abbreviations:** EIEC, enterotoxigenic *Escherichia coli*; PMXMP, polymyxin-mannitol-xylitol medium for *Providencia*; RAPD, random amplified polymorphic DNA.
(enterohemorrhagic *E. coli*, EHEC), *Klebsiella pneumoniae* RIMD 1102001, *Salmonella enteritidis* RIMD 1933006, *Shigella boydii* RIMD 1998008, *Proteus vulgaris* RIMD 1643003, *Proteus mirabilis* RIMD 1641002, *Morganella morganii* RIMD 1642003, *Pseudomonas aeruginosa* RIMD 1603014 and *Serratia liquefaciens* RIMD 3126001 were used for evaluation of the medium (Table 2). *P. alcalifaciens* RIMD 1656001, *Salmonella typhimurium* RIMD 1985089 and *E. coli* RIMD 05091207 (enteroinvasive *E. coli*, EIEC) were used as controls in the test of invasiveness.

**Preparation of PMXMP medium.** We modified *P. alcalifaciens* medium (PAM) (Senior, 1997), containing 10 g Lab Lemco powder (Oxoid), 5 g sodium deoxycholate, 0.8 g anhydrous disodium hydrogenphosphate, 80 mg phenol red dye and 12 g agar in 1 l distilled water, by adding sugars (maltose and xylose) separately sterilized at 100°C for 10 min, and polymyxin B sulfate (Pfizer) (250 000 units in 5 ml distilled water sterilized by filtration; pore size, 0.2 µm). After the base medium with these sugars was cooled to 50°C, polymyxin B was added.

**Evaluation of PMXMP.** Seven strains of *P. alcalifaciens*, three strains of *P. stuartii*, two strains of *P. rettgeri* and one strain of other species, including the strains listed in Table 2, were inoculated onto PMXMP to observe their growth. After 48 h incubation, colony formation and the colour of colonies were observed. Strains listed in Table 2 were inoculated into 1 ml Luria–Bertani (LB) broth and incubated overnight at 37°C with rotation. The dilution of cultures with PBS was done serially and 0.1 ml of diluent was plated on PMXMP and tryptic soy agar (TSA, Difco) as non-selective medium. The numbers of colony-forming units were counted on TSA after 24 h and on PMXMP after 48 h incubation.

**Identification of Providencia species.** The suspicious colonies coloured red to pink on PMXMP were mostly *Providencia* species or *M. morganii*, and occasionally *S. liquefaciens* and *Pseudomonas* species. The ornithine decarboxylase test distinguished *Providencia* species from *M. morganii* and *Serratia liquefaciens*, and the oxidase test distinguished *Providencia* species from *Pseudomonas* species. To determine the species among the genus of *Providencia*, the API 20E biochemical strip for the family *Enterobacteriaceae* (API System, BioMerieux) was used. The adonitol utility test helped to distinguish *P. alcalifaciens* and *P. rustigianii*. API 20E was also used to confirm *M. morganii*, *Serratia liquefaciens* and *Pseudomonas* species.

**Isolation of diarrhoeal pathogens from faeces at Kansai airport quarantine station.** In the Kansai airport quarantine station, faeces from patients with diarrhoea were usually inoculated onto *Salmonella–Shigella* agar (SS agar) (Nissui) and into alkaline peptone water. Cultures enriched for 6 h in alkaline peptone water were inoculated onto thiosulfate citrate bile sucrose agar (TCBS) (Nissui) to isolate *Vibrio cholerae* and other *Vibrio* species. From SS agar and TCBS, *Shigella* species, *Salmonella* species, *Plesiomonas* species, enteropathogenic *E. coli*, *Aeromonas* species, *Vibrio parahaemolyticus*, *V. cholerae*, *Vibrio fluvialis* and *Vibrio mimicus* could be isolated. For this study, faeces were also inoculated on PMXMP by the direct method. Enrichment of the culture (in alkaline peptone water) before the inoculation on PMXMP did not affect the detection of *Providencia* species. After
To determine the numbers of intracellular bacteria, we plated serial dilutions for 20 min at 37°C in 5% CO₂ at 8C with reciprocal shaking (150 r.p.m.). The bacterial cultures were diluted in PBS to remove non-invasive bacteria. DMEM supplemented with 10% FCS containing gentamicin (100 μg ml⁻¹) was then added to each well to kill any extracellular bacteria not removed by the PBS washes. After an additional 2 h incubation, the monolayers were lysed with 0.1% Triton X-100 in PBS for 20 min at 37°C with reciprocal shaking (150 r.p.m.). To determine the numbers of intracellular bacteria, we plated serial dilutions of the lysed monolayers in PBS on TSA (Merck) and incubated the samples overnight at 37°C. Assays were repeated three times, each time in duplicate. Results are shown as the percentage of bacteria internalized, which was calculated from the mean number of colony-forming units of the bacterial cells internalized in the Caco-2 cells and the number of bacteria in the original inoculum.

**Table 2. Growth of Providencia species and other species on PMXMP medium**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth*</th>
<th>c.f.u. ml⁻¹†</th>
<th>Colour of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TSA</td>
<td>PMXMP</td>
</tr>
<tr>
<td><em>Providencia alcalifaciens</em> RIMD 1656012</td>
<td>+</td>
<td>1·3 × 10⁸</td>
<td>4·1 × 10⁷</td>
</tr>
<tr>
<td><em>Providencia stuartii</em> RIMD 1660001</td>
<td>+</td>
<td>8·8 × 10⁸</td>
<td>2·3 × 10⁸</td>
</tr>
<tr>
<td><em>Providencia rettgeri</em> RIMD 1658001</td>
<td>+</td>
<td>1·1 × 10¹⁰</td>
<td>1·2 × 10⁸</td>
</tr>
<tr>
<td><em>Providencia rustigianii</em> RIMD 1659002</td>
<td>+</td>
<td>2·6 × 10⁸</td>
<td>1·6 × 10⁸</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (ETEC) RIMD 0509770</td>
<td>+</td>
<td>4·5 × 10⁸</td>
<td>3·0 × 10</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (EHEC) RIMD 0509952</td>
<td>+</td>
<td>2·3 × 10⁸</td>
<td>Less than 10</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> RIMD 1102001</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em> RIMD 1933006</td>
<td>+</td>
<td>3·2 × 10⁸</td>
<td>3·0 × 10</td>
</tr>
<tr>
<td><em>Shigella boydii</em> RIMD 1998008</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> RIMD 1643003</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> RIMD 1641002</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Morganella morganii</em> RIMD 1642003</td>
<td>+</td>
<td>4·0 × 10⁸</td>
<td>6·0 × 10⁵</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> RIMD 1603014</td>
<td>+</td>
<td>1·5 × 10¹¹</td>
<td>4·0 × 10³</td>
</tr>
<tr>
<td><em>Serratia liquefaciens</em> RIMD 3126001</td>
<td>+</td>
<td>1·9 × 10⁹</td>
<td>4·1 × 10⁸</td>
</tr>
</tbody>
</table>

*Incubated on PMXMP at 37°C for 48 h.
† c.f.u. on PMXMP after 48 h incubation and TSA after 24 h incubation. ND, Not done.

40 h at 37°C, red to pink colonies on PMXMP were collected. Suspicous colonies without ornithine decarboxylase activity were identified using the API 20E system (bioMérieux) and adonitol fermenting test. Surveillance was done for a period of 1 month (from 15 June 2002 to 14 July 2002) at the Kansai airport quarantine station. The number of travellers with diarrhoea during this period was 130 (all except one were adults). The majority of travellers who complained of diarrhoea were returning from South-East Asia.

**Healthy controls.** As it is hard to collect the stools of healthy returning travellers, we examined the stools of food handlers, who are obligated to regularly give stool samples to confirm that they are not infected by enteropathogens. During one month, 177 specimens were analysed by the PMXMP method.

**Invasion assay.** The invasion assay was conducted essentially as described elsewhere (Akeda et al., 1997). In brief, we grew Caco-2, a human colon carcinoma cell line, in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% CO₂ at 37°C. Assays were performed in 48-well tissue culture plates (Corning). For the invasion assay, cultures were grown to stationary phase at 37°C in LB broth with rotation. The bacterial cultures were diluted in PBS to give a m.o.i. of 1. Cell monolayers were challenged with bacterial suspensions for 3 h at 37°C in 5% CO₂ and then washed three times in PBS to remove non-invasive bacteria. DMEM supplemented with 10% FCS containing gentamicin (100 μg ml⁻¹) was then added to each well to kill any extracellular bacteria not removed by the PBS washes. After an additional 2 h incubation, the monolayers were lysed with 0·1% Triton X-100 in PBS for 20 min at 37°C with reciprocal shaking (150 r.p.m.). To determine the numbers of intracellular bacteria, we plated serial dilutions of the lysed monolayers in PBS on TSA (Merck) and incubated the samples overnight at 37°C. Assays were repeated three times, each time in duplicate. Results are shown as the percentage of bacteria internalized, which was calculated from the mean number of colony-forming units of the bacterial cells internalized in the Caco-2 cells and the number of bacteria in the original inoculum.

**Plasmid profiles.** Using the method of Birnboim & Doly (1979), we extracted plasmid DNA from overnight cultures of *P. alcalifaciens* strains and *P. rettgeri* strains, and examined them by electrophoresis in 0·5% agarose gels.

**Random amplified polymorphic DNA (RAPD) fingerprinting.** Genomic DNA was prepared from overnight cultures (1 ml) in LB broth at 37°C with a DNA purification kit (QIA amp DNA mini kit; QIAGEN KK) as recommended by the manufacturer. RAPD reactions with the genomic DNA preparations were performed using beads (Ready-To-Go RAPD Analysis Beads, Amersham Biosciences) according to the manufacturer’s directions. For the RAPD analysis, primers 3 (5’-d[GTAGACCCGT]-3’) and 5 (5’-d[AACGCGCAAC]-3’) were used. Amplification products were separated by electrophoresis in 2% agarose (Invitrogen) and visualized by ethidium bromide staining.

**RESULTS AND DISCUSSION**

Among Enterobacteriaceae, including Providencia species, *M. morganii* is the only species that does not ferment maltose-xyllose like Providencia species. As shown in Table 2, *E. coli* and *Salmonella* species could grow on PMXMP but their colonies were yellow, distinct from the red to pink colonies formed by *Providencia* species. The rate of growth inhibition (colony formation) on PMXMP for *E. coli* or *Salmonella* species was extremely high, about 10⁵–10⁷ times that for *Providencia* species. Since *M. morganii* formed colonies with a red to pink colour like *Providencia* species, we distinguished them by examining ornithine decarboxylase activity using Möller ornithine medium (Eiken kagaku): the former is positive and the latter negative for the activity.

As shown in Table 1, 23 strains of *Providencia* species were isolated on PMXMP from 20 patients (15·4%) among 130. In addition, other species were isolated by routine methods (SS agar and TCBS), including *Plesiomonas shigelloides* from...
13 patients (10%) and Vibrio parahaemolyticus from nine patients (6-9%) (Table 3). P. rettgeri was isolated in nine cases, P. alcalifaciens in nine cases and P. stuartii in five cases. Four of 20 subjects with Providencia species were also infected with some other species of enteric bacteria (patients 3, 6, 8 and 17 in Tables 1 and 3). Two different species of Providencia were isolated simultaneously in three cases (patients 5, 12 and 13 in Table 1). In the remaining Providencia cases, one Providencia species only was isolated.

The symptoms of patients from whom Providencia species were isolated are summarized in Table 1. Diarrhoea was seen in all subjects except one (patient 18), who complained of vomiting on the day the faeces were obtained. Seven of the 20 patients complained of abdominal pain and four patients had a fever. Vomiting was seen in five patients from whom P. rettgeri was isolated.

Among 177 specimens from healthy controls, six specimens (seven strains) were Providencia-positive (3-4%). Three strains of P. rettgeri, one strain of P. stuartii, one strain of P. alcalifaciens and two strains of P. rustigianii were isolated. One specimen contained both P. rustigianii and P. alcalifaciens.

Although nine P. rettgeri strains were isolated from the diarrhoeal patients in our case, P. rettgeri has not to our knowledge been reported previously as an enteropathogenic bacterium. To analyse the pathogenicity of these strains, we performed an invasion test using Caco-2 cells (Fig. 1). Four of the nine P. rettgeri strains and two of the nine P. alcalifaciens strains were highly invasive. Four P. rettgeri strains, three P. alcalifaciens strains and one P. stuartii strain showed weak invasive activity. As a positive reference, we used a P. alcalifaciens strain isolated previously in Japan (Murata et al., 2001), EIEC and Salmonella typhimurium; all were positive as expected (Fig. 1). None of seven strains from healthy controls showed invasive activity.

In some Enterobacteriaceae, a large plasmid, the virulence plasmid, has been implicated in the ability of the organism to invade eukaryotic cells (Hale, 1991). We analysed the plasmid profile of the P. alcalifaciens and P. rettgeri strains, and found that all strains had a large (>100 kbp) plasmid (data not shown). We could not find, however, any relationship between invasiveness in Caco-2 cells and the possession of a common plasmid in the P. rettgeri strains.

Nine strains of P. rettgeri isolated from travellers with
diarrhoea returning from various countries of South-East Asia were analysed further for genetic relatedness. Among the travellers from whom P. rettgeri was isolated, patients 5 (RIMD 1658019) and 7 (RIMD 1658023) (Table 1) belonged to the same tour group. These two strains showed the same genotype on RAPD fingerprinting (Fig. 2). The results strongly suggest that patients 5 and 7 were infected with P. rettgeri abroad and developed diarrhoea, and that the strains were not commensals in the gastrointestinal tract.

Our results showed that PMXMP medium is an excellent selective medium for the isolation of Providencia species. One can not distinguish Providencia species from other non-lactose-fermenters such as Salmonella species or Shigella species using DHL, MacConkey agar or SS agar as a selective medium for the isolation of bacteria from faeces. Testing at the Kansai airport quarantine station using PMXMP as a selective medium for Providencia species showed a high incidence of Providencia species in diarrhoeal faeces. Since faeces from diarrhoeal patients are usually inoculated into alkaline peptone water to enrich V. cholerae at the quarantine station, we also used alkaline peptone water in this study. We obtained the same results with the direct method and the enrichment method using alkaline peptone. Of 20 patients from whom Providencia species were isolated, other enteropathogens were also isolated in four, but only Providencia species were isolated in the remaining 16. Although P. alcalifaciens seems to be an enteropathogen (Albert et al., 1998; Guth & Ferrella, 1996; Haynes & Hawkey, 1989; Murata et al., 2001), P. rettgeri and P. stuartii have not been reported to cause human diarrhoea until now. Furthermore, most of the P. rettgeri strains showed invasive activity in Caco-2 cells but P. stuartii did not. These results suggest that P. rettgeri is a diarrhoea-causing pathogen.

Providencia species are likely to be pathogens for diarrhoea among Japanese travellers abroad. This is in agreement with the report of Haynes & Hawkey (1989) in Britain, who analysed the relationship between P. alcalifaciens and travellers’ diarrhoea. We isolated not only P. alcalifaciens but also P. stuartii and P. rettgeri from travellers with diarrhoea. Some of the patients infected with Providencia species complained of abdominal pain, fever and vomiting. As detailed information on clinical symptoms is not available, the relation between invasive activity and clinical symptoms is not clear. Vomiting was a rather characteristic complaint among patients with diarrhoea caused by P. rettgeri. Although the cause of the vomiting is not clear yet, another virulence factor like emetic toxin might be produced by P. rettgeri strains.

The virulence plasmid possessed by Shigella species and EIEC has been implicated in the ability of the organism to invade eukaryotic cells (Hale, 1991). Guth & Ferrella (1996) reported that the invasive ability of P. alcalifaciens strains in HeLa cells was not related to the possession of a plasmid, but our previous results suggested that a large plasmid might...
in some way be related to the invasiveness of *P. alcalifaciens* strains in Caco-2 cells (Murata *et al.*, 2001). Here we could not find any relationship between invasiveness of *P. rettgeri* in Caco-2 cells and the possession of a (large) virulent plasmid. *P. rettgeri* might carry invasion-associated genes chromosomally like other enteric pathogens.

The epidemiological typing of *P. rettgeri* isolates by RAPD fingerprinting was also performed. Two strains from patients who travelled together showed the same genotype on RAPD fingerprinting, while all the other strains differed. As the two patients were not related and were not even living in the same household, it is not possible that they were exposed to the same pathogen in their daily life. The finding suggests that these two individuals became infected with *P. rettgeri* while abroad. As we cannot rule out that parasitic or viral agents were associated with the travellers’ diarrhoea, further analysis is required.

This is the first report that suggests that *P. rettgeri* is an enteropathogen in adults. We believe that *P. rettgeri* is a cause of diarrhoea among travellers returning to Japan. The mechanism behind the diarrhoea caused by *P. rettgeri* may be invasiveness but further study is necessary.

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**REFERENCES**


