Identification and Characterization of *Yersinia intermedia* Isolated from Human Feces

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Since May 1983, our laboratory has, upon request, cultured stools for *Yersinia* spp. by using direct plating on cefsulodin-irgasan-novobiocin agar and a 3-week cold enrichment procedure. We isolated bacteria identified as *Y. intermedia* from six adult patients. All isolates were recovered only by the cold enrichment procedure and misidentified as *Y. enterocolitica* by the API 20E system (Analytab Products, Plainview, N.Y.). Final identification was made on the basis of results obtained with conventional tube biochemical tests. The isolates were tested for the following characteristics associated with virulence in *Y. enterocolitica*: lack of pyrazinamidase activity, autoagglutinability, presence of a 40- to 50-megadalton plasmid, production of heat-stable enterotoxin, and mouse lethality. All isolates tested had pyrazinamidase activity, and none were autoagglutinable. However, one isolate possessed a 40-megadalton plasmid. None produced enterotoxin or were lethal for mice. Review of the medical histories of the patients revealed that four of the six had diarrhea; however, none had disease typical of that caused by *Y. enterocolitica*. Our data confirmed the limited pathogenic potential of *Y. intermedia* and suggested that its isolation was without clinical significance in our patients. Conventional biochemical tests were required for reliable identification of *Y. intermedia*.

*Yersinia intermedia* was defined biochemically and genetically as a species in 1980 (7). Acid production from α-methyl-D-glucoside, D-melibiose, D-raffinose, and L-rhamnose and utilization of citrate help distinguish it from *Y. enterocolitica* and other *Yersinia* species.

The pathogenic potential of *Y. intermedia* for humans has not been well defined, and there are few reports of its isolation from clinical specimens. Bottone et al. (5) isolated bacteria subsequently identified as *Y. intermedia* from nonmesenteric sources in 12 patients. Of the 321 strains of *Y. intermedia* studied by Brenner et al. (7), only 13% were of human origin. Butler et al. (8) described two patients from whom *Y. intermedia* was recovered from stools collected along with other enteric pathogens. A recent report by Agbonlahor (1) suggested that *Yersinia* strains biochemically mimicking *Y. intermedia* were responsible for four cases of acute diarrheal disease in humans.

Although *Y. intermedia* is usually considered nonpathogenic, it is possible that it may become host adapted and clinically significant for humans. Since May 1983, our laboratory has isolated *Y. intermedia* from the stools of six adult patients. Here we report our experience with the isolation, identification, and biological characterization of these strains.

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MATERIALS AND METHODS

Isolation and identification of *Yersinia* sp. From May 1983 through December 1986, 860 stools were cultured for *Salmonella*, *Shigella*, and *Campylobacter* species by accepted methods and for *Yersinia* species by direct plating on cefsulodin-irgasan-novobiocin agar (20) and cold enrichment in phosphate buffer (10). Cefsulodin-irgasan-novobiocin agar plates were incubated at room temperature for 48 h. A portion of stool was also suspended in 5 ml of phosphate buffer and refrigerated at 4°C for 3 weeks, at which time the stool suspension was plated on cefsulodin-irgasan-novobiocin agar. Mannitol-fermenting (red) colonies (11) were identified by the API 20E system (Analytab Products, Plainview, N.Y.) and conventional biochemical tests (7). Duplicate API 20E strips were incubated at 22 and 35°C and read after 24 and 48 h of incubation. Duplicate sets of biochemical tests were incubated at 22 and 35°C for 7 days. Identification of strains as *Y. intermedia* was confirmed by M. Shayegani, New York State Department of Health Laboratories, Albany.

Pyrazinamidase activity. The method of Kandolo and Wauters (14) was used for detection of pyrazinamidase activity. Trypticase soy agar slants containing 0.1% pyrazincarboxamide were inoculated and then incubated at 25°C for 48 h. Fresh 1.0% ferrous ammonium sulfate (1 ml) was added to the surface of the slants, and the reactions were noted after 15 min. Development of a pink color indicated the presence of pyrazinoic acid and was considered a positive test.

Autoagglutination. Bacteria were examined for autoagglutination by the method of Prpic et al. (19). Briefly, isolates were grown overnight in tryptone-yeast extract broth at 37°C and washed once in phosphate-buffered saline, and the suspension was adjusted to a milky consistency. Duplicate tubes containing 2 ml of the adjusted suspension were incubated at 25 and 37°C with shaking. Strains that agglutinated at 37 but not 25°C after 1 h of incubation were considered positive.

Plasmid analysis. Plasmid DNA was isolated by the method of Kado and Liu (13) from overnight broth cultures grown at 25°C. Plasmid preparations were subjected to electrophoresis in 0.7% agarose gels at 4 V/cm for 4 h. Molecular mass estimates were made relative to the follow-
Fermentation was shown at 22°C.

**Analytical temperatures** were determined after 48 h of incubation at 35°C.

**Enterotoxin assay.** Strains were grown at 25°C for 48 h in Trypticase (BBB Microbiology Systems, Cockeysville, Md.) soy broth. A sucking mouse assay (2) for heat-stable enterotoxin was performed on culture supernatants by J. Agbonlahor, Department of Microbiology and Immunology, Kirksville College of Osteopathic Medicine, Kirksville, Mo.

**Mouse lethality test.** Pairs of adult male ICR mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), weighing 19 to 21 g, were inoculated intraperitoneally with 5 × 10⁷ CFU in 0.5 ml. The inoculum was prepared from bacteria grown overnight in brain heart infusion broth at 25°C. The cells were pelleted and suspended in sterile saline to a turbidity equal to a 0.5 McFarland standard. The inoculum was confirmed by agar plate colony counts. The mice were observed daily for 21 days for signs of illness or death.

**Patients.** The medical records of those patients for whom diarrhea was a presenting symptom were reviewed. The following clinical features were noted: concomitant illness, duration of diarrhea, temperature of >38°C, nausea or emesis, abdominal cramps, blood or mucus in the stool, and arthralgias.

**RESULTS**

From May 1983 through December 1986 we isolated *Y. intermedia* from 6 (0.7%) of 860 stool cultures. All strains were recovered only by cold enrichment as mannitol-fermenting colonies on cefsulodin-irgasan-novobiocin agar. The API 20E and conventional biochemical identifications are shown in Table 1. All API 20E identifications were obtained from the Analytical Profile Index, 9th ed. The profile numbers determined after 24 h at both incubation temperatures were all excellent identifications for *Y. enterocolitica* (data not shown). Four different profile numbers were generated after incubation for 48 h. At 35°C, five of six profile numbers were excellent identifications, and one of six was a very good identification for *Y. enterocolitica*. At 22°C, four of six profile numbers were excellent identifications for *Y. enterocolitica*, and two of six were not listed in the Analytical Profile Index. Melibiose fermentation, as demonstrated by API 20E strips, was negative for four of six strains at 22°C and for all of the strains at 35°C. API 20E rhamnose fermentation and citrate utilization tests were negative for all strains at both temperatures.

The conventional biochemical tests identified all six strains as *Y. intermedia*. The results of the biochemical tests used to differentiate *Yersinia* spp. are shown in Table 1. All strains produced acid from α-methyl-D-glucoside and used citrate as a carbon source. Strain 2 failed to produce acid from rhamnose, and strain 4 did not ferment rhamnose and melibiose. On the basis of these reactions, strains 1, 3, 5, and 6 were assigned to biotype 1, and strain 2 was assigned to biotype 4. Although strain 1 resembled *Y. intermedia* by biochemical tests, it did not conform to any of the eight biotypes described by Brenner et al. (7). However, the biochemical pattern for strain 4 was identical to that proposed for *Y. intermedia* biotype 9 by Agbonlahor (1). The *Y. intermedia* strains were tested for the following phenotypic characteristics associated with virulence of *Y. enterocolitica*: lack of pyrazinamidase activity, autoagglutinability, presence of a 40 to 50-MDa plasmid, production of heat-stable enterotoxin, and mouse lethality. In each of the assays at least one *Y. enterocolitica* isolate positive for the test characteristic was included as a control. All *Y. intermedia* isolates tested possessed pyrazinamidase activity, and none were autoagglutinable. Strain 3 was found to harbor a 40-MDa plasmid that comigrated with a plasmid obtained from a recent clinical isolate of *Y. enterocolitica*, as well as a 2.7-MDa plasmid. None of the *Y. intermedia* isolates were found to produce a positive response in the sucking-mouse assay for heat-stable enterotoxin. Except for a single mouse inoculated with strain 5, all mice inoculated intraperitoneally with 5 × 10⁷ *Y. intermedia* survived. This mouse died of apparently natural causes since postmortem cultures of the peritoneal cavity and spleen failed to grow *Y. intermedia*. None of the surviving mice had any obvious signs of illness at the end of the 21-day observation period. Diarrhea was a presenting symptom in four of six patients from whose stool samples *Y. intermedia* was recovered. The clinical features of these four patients are presented in Table 2. Patients 3, 4, and 5 were hospitalized and had complicated hospital courses. Patient 2 was seen as an outpatient at a university health service. The duration of diarrhea in these patients ranged from 1 day to 2 years. Only patient 4 complained of abdominal cramps, and none were found to have blood or mucus in the stool. Only stools from patient 3 were examined for fecal leukocytes, and they were negative. Patient 3 was also the only patient to have *Y. intermedia* isolated from two consecutive stool cultures. Patient 5 complained of pain in both the right and left knees. However, on examination, there was no swelling or joint stiffness found. Stools from patients 3, 4, and 5 were examined for ova and parasites and were negative. The isolates obtained from patients 1 and 6 were from formed stools. Patient 1 was a healthy control evaluated as part of an epidemiologic investigation of a foodborne out-

### Table 1. API 20E and conventional biochemical identifications of six clinical isolates of *Y. intermedia*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>API profile no. at 22°C</th>
<th>API identificationa</th>
<th>Citrate (Simmons)</th>
<th>l-Rhamnose</th>
<th>l-Raffinose</th>
<th>d-Melibiose</th>
<th>α-Methyl-D-glucoside</th>
<th>Final identification</th>
<th>Biotypeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1155763 1155723</td>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td><em>Y. intermedia</em></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1154723 0155273</td>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td><em>Y. intermedia</em></td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>1155763 1155723</td>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td><em>Y. intermedia</em></td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1155723 1154723</td>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td><em>Y. intermedia</em></td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>1155723 1155723</td>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td><em>Y. intermedia</em></td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1154723 1155723</td>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td><em>Y. intermedia</em></td>
<td>1</td>
</tr>
</tbody>
</table>

a. After 48 h of incubation.

b. Determined after 48 h of incubation at 35°C.

c. Conventional biochemical tests incubated for 7 days at 22°C.

d. Biotypes as described by Brenner et al. (7) and Agbonlahor (1).

ing standard plasmids: R40a, 90 megadaltons (MDa); R64, 72.3 MDa; and *Escherichia coli* V517 plasmids, 1.4 to 35.8 MDa.

**Enterotoxin assay.** Strains were grown at 25°C for 48 h in Trypticase (BBB Microbiology Systems, Cockeysville, Md.) soy broth. A sucking mouse assay (2) for heat-stable enterotoxin was performed on culture supernatants by J. Agbonlahor, Department of Microbiology and Immunology, Kirksville College of Osteopathic Medicine, Kirksville, Mo.

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**Patients.** The medical records of those patients for whom diarrhea was a presenting symptom were reviewed. The following clinical features were noted: concomitant illness, duration of diarrhea, temperature of >38°C, nausea or emesis, abdominal cramps, blood or mucus in the stool, and arthralgias.

**RESULTS**

From May 1983 through December 1986 we isolated *Y. intermedia* from 6 (0.7%) of 860 stool cultures. All strains were recovered only by cold enrichment as mannitol-fermenting colonies on cefsulodin-irgasan-novobiocin agar. The API 20E and conventional biochemical identifications are shown in Table 1. All API 20E identifications were obtained from the Analytical Profile Index, 9th ed. The profile numbers determined after 24 h at both incubation temperatures were all excellent identifications for *Y. enterocolitica* (data not shown). Four different profile numbers were generated after incubation for 48 h. At 35°C, five of six profile numbers were excellent identifications, and one of six was a very good identification for *Y. enterocolitica*. At 22°C, four of six profile numbers were excellent identifications for *Y. enterocolitica*, and two of six were not listed in the Analytical Profile Index. Melibiose fermentation, as demonstrated by API 20E strips, was negative for four of six strains at 22°C and for all of the strains at 35°C. API 20E rhamnose fermentation and citrate utilization tests were negative for all strains at both temperatures.

The conventional biochemical tests identified all six strains as *Y. intermedia*. The results of the biochemical tests
TABLE 2. Clinical features of four patients with diarrhea from whose feces Y. intermedia was isolated

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>19</td>
<td>86</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Concomitant illness</td>
<td>None</td>
<td>Congestive heart failure</td>
<td>Restrictive lung disease</td>
<td>None</td>
</tr>
<tr>
<td>Duration of diarrhea</td>
<td>24 h</td>
<td>6 wk</td>
<td>2 yr</td>
<td>48 h</td>
</tr>
<tr>
<td>Temperature of &gt;38°C</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Nausea or emesis</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Abdominal cramps</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Blood in stool</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Mucus in stool</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Discharge diagnosis</td>
<td>Probable viral gastroenteritis</td>
<td>Diarrhea of uncertain etiology</td>
<td>Irritable-bowel syndrome</td>
<td>Fever of unknown origin</td>
</tr>
</tbody>
</table>

* Patient numbers correspond to strain numbers in Table 1.

break of viral gastroenteritis. Patient 6 was the father of an infant who suffered an acute gastrointestinal disease of uncertain etiology.

**DISCUSSION**

The six strains of *Y. intermedia* included in this study were all misidentified as *Y. enterocolitica* by the API 20E system. Delayed utilization of rhamnose, melibiose, and citrate probably accounted for these misidentifications. Our results and those of Baker and Farmer (3) demonstrate that reliable identification of isolates identified as *Y. enterocolitica* by the API 20E systems requires further testing by conventional biochemical methods. Although the API data base includes the "newer" *Yersinia* species hitherto referred to as atypical *Y. enterocolitica* or *Y. enterocolitica*-like bacteria, our data indicate that larger numbers of *Y. intermedia* isolates need to be tested to improve the ability of the system to distinguish the species.

One of our isolates of *Y. intermedia*, strain 4, did not conform to any of the eight biotypes described by Brenner et al. (7). This strain resembles *Y. enterocolitica* in its abilities to ferment rhamnose and α-methyl-D-glucoside and use citrate as a carbon source. However, it failed to produce acid from melibiose and raffinose even after incubation for 14 days. Shayegani et al. (21) described a similar strain isolated from a water sample submitted from Essex County, N.Y. Agbonlahor (1) also reported biochemically similar strains isolated from the feces of five patients with acute diarrhea disease in Lagos, Nigeria. He suggested the strains with the above biochemical pattern be referred to as *Y. intermedia*-like or *Y. intermedia* biotype 9 until their taxonomic status is clarified.

The clinical significance of *Y. intermedia* and the other newly described *Yersinia* species is not well defined. *Y. intermedia* has been isolated from all parts of the world, with the majority of strains originating from environmental or animal sources (7, 21). *Y. intermedia* has only rarely been reported from human stool specimens but has been associated with human wound infections (4). We chose to assess the pathogenic potential of our *Y. intermedia* isolates by using assays that have been reported to be indicators of virulence for *Y. enterocolitica*.

Kandolo and Wauters (14) reported that the pyrazinamidase test was a valuable tool to distinguish potentially pathogenic from nonpathogenic strains of *Yersinia* sp. The sxy *Y. intermedia* strains described in our study were all positive in the pyrazinamidase test, unlike pathogenic strains of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis.*

Autoagglutinability is commonly used as an indicator of *Yersinia* virulence for mice (15, 16). A positive autoagglutination test is highly predictive of mouse lethality; however, a nonautoagglutinable, lethal strain has been described (19). None of the *Y. intermedia* strains examined by Prpic et al. (19) and Kay et al. (15) were autoagglutinable. In contrast to these findings, Agbonlahor (1) reported that four of five *Y. intermedia* biotype 9 strains autoagglutinated. None of the isolates of *Y. intermedia* obtained from our patients were autoagglutinable, including our one biotype 9 strain.

The presence of a 40- to 50-MDa plasmid determines virulence in *Y. enterocolitica* (9, 18). However, since both chromosome- and plasmid-determined properties are required for full expression of pathogenic potential, the presence of a plasmid of the appropriate size class is a necessary but not sufficient condition for virulence (12). The *Y. intermedia* isolate from patient 3 harbored a 40-MDa plasmid. Prpic et al. (19) found that six of eight *Y. intermedia* isolates possessed plasmids of 40 to 50 MDa. None of the five *Y. intermedia* biotype 9 strains examined by Agbonlahor (1) contained plasmids of this molecular mass. Although more strains need to be tested, virulence plasmids apparently are not uncommon in *Y. intermedia*.

Pai and Mors (17) reported that all of the 43 clinical isolates of *Y. enterocolitica* they examined were enterotoxigenic when tested in the infant-mouse model. This enterotoxin was shown to be similar to *E. coli* heat-stable enterotoxin. Kay et al. (15) reported that only 10 of 100 *Y. enterocolitica* and 1 of 10 *Y. intermedia* isolates produced a heat-stable enterotoxin. Although the clinical relevance of enterotoxin production by *Yersinia* sp. has not been determined, a poor correlation exists between enterotoxin production and mouse lethality (15). None of our *Y. intermedia* strains was found to elaborate an enterotoxin active in the infant-mouse model. Production of heat-labile toxin by *Y. enterocolitica* or other *Yersinia* species has not been described.

An appropriate animal model that determines the capacity of an organism to multiply in vivo, invade tissues, and produce disease and ultimately death is probably the best reflection of the ability of an organism to produce disease in humans. None of the *Y. intermedia* isolates we tested were lethal for mice after intraperitoneal inoculation of 5 × 10⁷ CFU. Kay et al. (15), using an inoculum of 5 × 10⁶ CFU, showed that none of 10 *Y. intermedia* isolates were lethal for mice. Prpic et al. (19) inoculated iron-overloaded mice with 10⁷ CFU of eight different *Y. intermedia* strains and found that all of the mice survived. Likewise, Agbonlahor (1) found
that none of the Y. intermedia biotype 9 strains he examined were lethal for mice. These studies demonstrated that Y. intermedia, unlike Y. enterocolitica O:8, has a limited capacity for in vivo replication in mice.

It was clear from a review of the medical histories that isolation of Y. intermedia from patients 1 and 6 was without clinical significance. Neither patient had gastrointestinal tract symptoms, and the organisms were recovered from surveillance cultures. Although the remaining four patients from whom we recovered Y. intermedia had diarrhea as a presenting symptom, the clinical features were not those typically described for Yersinia infections (6). Patient 5 complained of transitory left- and right-knee pain concurrent with her diarrhea; however, no joint swelling or stiffness was found. In most cases of Y. enterocolitica-induced polyarthritis, multiple joints become inflamed in rapid succession over a period of 2 to 14 days. Symptoms persist for months in most cases.

The etiology of the diarrhea in our four patients was never firmly established. The fact that cold enrichment was necessary for isolation of Y. intermedia from all of our patients, despite direct plating of feces on medium selective for yersinae, indicated that they were shed in small numbers. Based on the clinical features and in vitro and in vivo assessments of virulence, it seems unlikely that Y. intermedia was involved in the pathogenesis of diarrhea in these patients. However, our data do not completely exclude this possibility.

Important differences in pathogenic potential exist between Y. enterocolitica and Y. intermedia. Because of these differences it is important to distinguish these species reliably. Precise definition and complete biological characterization of Yersinia spp. by clinical microbiologists should help clarify what role, if any, Y. intermedia plays in the pathogenesis of human diarrheal disease.

ACKNOWLEDGMENTS

We thank M. Shayegani for confirming the identities of the strains, F. Lyon for performing the enterotoxin assays, and C. Zulick for excellent technical assistance.

LITERATURE CITED