

## Characteristics of *Yersinia enterocolitica* Isolated from Children with Diarrhea in Italy

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Of 2,500 fecal samples collected from children with diarrhea in the province of Siena, 35 (1.4%) were found to be positive for *Yersinia enterocolitica*. Of the isolates, 94.2% belonged to biotype 4, serotype O:3; 2.8% belonged to biotype 2, serotype O:9; and 2.8% belonged to biotype 1, serotype O:6. The in vitro pathogenicity tests showed that all but two isolates were calcium dependent and autoagglutinable and that all but one were also invasive in HEP-2 cell culture. As regards plasmid content, 32 of 33 biotype 4, serotype O:3 strains harbored a plasmid of 48 megadaltons and 1 strain also harbored a small plasmid of 2 megadaltons. The biotype 2, serotype O:9 strain harbored a plasmid of 42 megadaltons; one of the two strains lacking plasmids belonged to biotype 1, serotype O:6, and the other belonged to biotype 4, serotype O:3. Pyrazinamidase activity was positive only for the biotype 1, serotype O:6 strain. Esculin was hydrolyzed only by the biotype 1, serotype O:6 strain.

*Yersinia enterocolitica* causes a variety of human infections, ranging from mild enterocolitis, mesenteric lymphadenitis, and arthritis to severe septicemia; children and persons whose host defense mechanisms are compromised appear to be the groups at risk for septicemia. The most common clinical picture is an acute diarrheal disease (4, 13). Approximately 90% of the cases are self-limiting, although complications in immunodeficient hosts have been described (19).

The serotypes most commonly isolated from humans and considered to be human pathogens are O:3, O:8, and O:9 (30). The virulent strains harbor a plasmid DNA species ranging from 40 to 50 megadaltons (MDa) in size (7, 19, 21). This plasmid, when present, determines a number of characteristics, most of which are expressed phenotypically only at temperatures exceeding 30°C.

The purpose of the present study was to verify the importance of *Y. enterocolitica* as an etiological agent of diarrhea in children in the province of Siena, Italy. The monthly and seasonal incidence of *Y. enterocolitica* isolates was studied. A number of pathogenicity tests both in vivo and in vitro were carried out on the isolates.

### MATERIALS AND METHODS

**Study population.** A total of 2,500 fecal samples from children with diarrhea in the province of Siena were examined during the period January 1981 to December 1985. Of these children, 575 were patients admitted with diarrhea to the Institute of Infectious Diseases in Siena and 1,925 were outpatients. All children were aged 14 years or less (mean age, 3.5 years).

The symptoms ranged from mild diarrhea with or without mucus and with or without blood in feces to fever and vomiting in a large number of cases; abdominal pain did not occur frequently.

**Stool examination.** Stool samples were cultured for *Yersinia* sp. directly onto MacConkey agar with Tween 80

(14) and salmonella-shigella agar with sodium desoxycholate (27) and incubated at 28°C for 48 h.

Phosphate-buffered saline (pH 7.6) was also inoculated for cold enrichment and subcultured onto the same media used for the direct plating after 1 and 3 weeks of incubation at 4°C.

**Biochemical and serological identification.** Identification of the strains was performed by biochemical tests with the API 20 E system; furthermore, the reactions to 49 carbohydrates were tested by using the API 50CH system. The cultures were incubated at 28°C and observed for 1 week. The biotype was determined by using the scheme of Bercovier et al. (3). Serological typing was performed by the method of Wauters et al. (28, 29).

**Weather data.** Weather data were provided by the Meteorological Service of the Seismic Observatory of Siena. Mean temperature and humidity values were deduced from daily mean values, calculated by four recordings per day, and expressed as degrees Celsius and percent humidity, respectively.

**Antibiotic susceptibility.** Susceptibility to antimicrobial agents was determined by the agar disk diffusion method (1), with the following antibacterial drugs: ampicillin (10 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), colistin (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), tobramycin (10 µg), trimethoprim-sulfamethoxazole (1.25 and 23.75 µg), tetracycline (30 µg), streptomycin (10 µg), and sulfisoxazole (2.0 mg).

**Pyrazinamidase activity.** Pyrazinamidase activity was detected by the method of Kandolo and Wauters (10). Hydrolysis of pyrazinamide by pyrazine carboxylamidase resulted in the production of pyrazinoic acid, which turned brownish pink in the presence of ferrous salts.

**Calcium dependency.** The strains were grown for 20 h at 37°C on Trypticase soy agar (BBL Microbiology Systems) and on magnesium oxalate agar (8, 23). Growth on the two plates was compared: growth on Trypticase soy agar and inhibition of growth (fewer numbers or markedly smaller colonies) on magnesium oxalate agar was considered to be a

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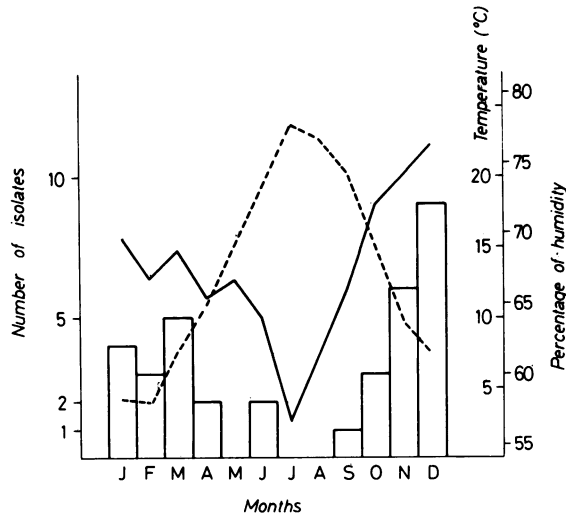


FIG. 1. Cumulative monthly incidence of human *Y. enterocolitica* infections (□) and temperature (----) and humidity (—) in Siena from 1981 to 1985.

positive test (CAD<sup>+</sup>). Equal numbers of similar-sized colonies on both media were indicative of a negative test (CAD<sup>-</sup>).

**Autoagglutination.** The strains were examined for their ability to undergo autoagglutination in Eagle minimal essential medium containing 10% fetal calf serum and 1 mg of sodium bicarbonate per ml (11, 12). At 37°C, autoagglutination-positive bacteria formed a flocculate covering the bottom of the tube, leaving a clear supernatant, whereas at 22°C, uniform turbid growth was observed. Autoagglutination-negative bacteria produced turbid growth at both temperatures.

**Test for invasiveness.** The in vitro test for invasiveness with HEP-2 cell cultures was carried out as described previously (5,16).

**LD<sub>50</sub>.** The 50% lethal dose (LD<sub>50</sub>) in mice was determined by the method described by Smith et al. (24). The LD<sub>50</sub> was calculated from cumulative mortality data by the method of Reed and Muench (22).

**Plasmid analysis.** Plasmid extraction and electrophoresis were performed as described by Kado and Liu (9). The molecular size of the plasmid was estimated by comparing electrophoretic mobilities with those of plasmids of a known

molecular size (17), including R27 (112 MDa), R16 (69 MDa), R471 (52 MDa), RP4 (36 MDa), and pBR322 (2.84 MDa).

**RESULTS**

Of the 2,500 patients examined from 1981 to 1985, 35 (1.4%) had samples that were positive for *Yersinia* sp. All the isolates grown by direct culture were *Y. enterocolitica*. The percentage of positive samples isolated for each year was as follows: 4.4% in 1981, 0.9% in 1982, 0% in 1983, 1.3% in 1984, and 2.5% in 1985.

Of the 35 cases of yersiniosis, 29 (82.9%) occurred between November and April, and 6 (17.1%) occurred between May and October. During the period considered, the number of isolations of *Y. enterocolitica* was higher from November to March, when the mean temperature was lower and the mean humidity was higher (Fig. 1).

Of the 35 isolates, 33 (94.2%) belonged to biotype 4, serotype O:3, 1 (2.8%) belonged to biotype 2, serotype O:9, and 1 (2.8%) belonged to biotype 1, serotype O:6.

Table 1 reports the results concerning antimicrobial susceptibility, biochemical characteristics, pathogenicity tests, and plasmid content for all the isolates. All 35 strains were found to be resistant to the beta-lactam antibiotics ampicillin and cephalothin, and of these, 2 strains were also found to be resistant to cefoxitin, 2 were resistant to streptomycin, and 3 were resistant to streptomycin and sulfisoxazole. Pyrazinamidase activity was positive only for isolates of biotype 1, serotype O:6. Within 48 h, esculin was hydrolyzed only by the biotype 1, serotype O:6 strain. All the other isolates were negative, except the biotype 2, serotype O:9 strain and two biotype 4, serotype O:3 strains, which showed a delayed reaction after several days.

Tests for virulence-associated phenotypic markers performed in vitro showed that calcium dependency and autoagglutination occurred for all but two isolates and that all but one were also invasive in HEP-2 cells.

Of seven isolates tested for the LD<sub>50</sub> in mice, two isolates, one of biotype 1, serotype O:6 and the other of biotype 4, serotype O:3, had an LD<sub>50</sub> higher than 10<sup>7</sup> CFU/ml, while four strains of biotype 4, serotype O:3 and one of biotype 2, serotype O:9 had an LD<sub>50</sub> lower than 10<sup>7</sup> CFU/ml.

With regard to plasmid content, 32 strains of biotype 4, serotype O:3 harbored a plasmid of 48 MDa; of these, 1 strain also harbored a small plasmid of 2 MDa. The biotype 2, serotype O:9 strain harbored a plasmid of 42 MDa. Two strains were reported to lack plasmids; one was of biotype 1,

TABLE 1. Characteristics of *Y. enterocolitica* isolates from children with diarrhea in Siena

No. of strains	Biotype	Serotype	Resistance <sup>a</sup>	Pyrazinamidase activity	Esculin hydrolysis	Calcium dependency	Autoagglutination	HEP-2 invasiveness	LD <sub>50</sub> in mice	Plasmid size (MDa)
1	1	O:6	Am <sup>r</sup> Cf <sup>r</sup> Fox <sup>r</sup>	+	+	-	-	-	>6.3 × 10 <sup>7</sup>	
1	2	O:9	Am <sup>r</sup> Cf <sup>r</sup> Fox <sup>r</sup>	-	-	+	+	+	6.6 × 10 <sup>6.8</sup>	42
1	4	O:3	Am <sup>r</sup> Cf <sup>r</sup>	-	-	-	-	+	>4.5 × 10 <sup>7</sup>	
1	4	O:3	Am <sup>r</sup> Cf <sup>r</sup>	-	-	+	+	+	4.8 × 10 <sup>6.7</sup>	48
1	4	O:3	Am <sup>r</sup> Cf <sup>r</sup>	-	-	+	+	+	6.2 × 10 <sup>6.5</sup>	48
1	4	O:3	Am <sup>r</sup> Cf <sup>r</sup>	-	-	+	+	+	4.2 × 10 <sup>6.4</sup>	48
24	4	O:3	Am <sup>r</sup> Cf <sup>r</sup>	-	-	+	+	+	NT <sup>b</sup>	48
2	4	O:3	Am <sup>r</sup> Cf <sup>r</sup> S <sup>r</sup>	-	-	+	+	+	NT	48
1	4	O:3	Am <sup>r</sup> Cf <sup>r</sup> S <sup>r</sup> G <sup>r</sup>	-	-	+	+	+	7.7 × 10 <sup>6.8</sup>	48; 2
2	4	O:3	Am <sup>r</sup> Cf <sup>r</sup> S <sup>r</sup> G <sup>r</sup>	-	-	+	+	+	NT	48

<sup>a</sup> Am<sup>r</sup>, Ampicillin resistance; Cf<sup>r</sup>, cephalothin resistance; Fox<sup>r</sup>, cefoxitin resistance; G<sup>r</sup>, sulfisoxazole resistance; S<sup>r</sup>, streptomycin resistance.

<sup>b</sup> NT, Not tested.

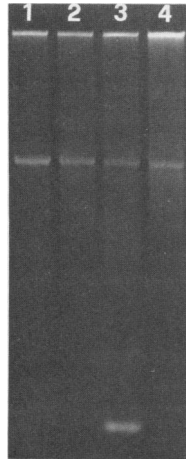


FIG. 2. Agarose gel electrophoresis (0.7% agarose) of plasmid DNA from strains of *Y. enterocolitica*. Lanes: 1 and 2, biotype 4, serotype O:3 (48 MDa); biotype 4, serotype O:3 (48 and 2 MDa); 4, biotype 2, serotype O:9 (42 MDa).

serotype O:6 and the other was of biotype 4, serotype O:3. Figure 2 shows the three representative plasmid profiles.

#### DISCUSSION

The bioserotypes of *Y. enterocolitica* most frequently isolated from patients with enteritis in Europe are biotype 4, serotype O:3 and biotype 2, serotype O:9 (30).

In agreement with these data, during the investigation we carried out in the area of Siena on 2,500 children with diarrhea, we found biotype 4, serotype O:3 to account for 94.2% of the total isolations of *Y. enterocolitica*. This bioserotype has also been recovered from animals such as pigs (18, 30) and dogs (6, 18), but the zoonotic significance, as well as the mechanism of transmission from animals to humans, has yet to be clarified.

Our data also point out that the frequency of isolation was not very homogeneous over the years. In 1981 the percentage of isolates was high because only hospitalized patients were examined. The lack of isolations in 1983 is inexplicable. The total frequency of isolation of *Y. enterocolitica* strains was 1.4%, confirming that in the area considered, enteritis caused by this organism is not particularly frequent.

In agreement with other authors (2, 15, 25), we noted a higher frequency of *Y. enterocolitica* isolates in the autumn and winter, when the weather is colder and more humid.

The antimicrobial resistance pattern showed few multiresistant strains. Although such strains are not found with a high frequency, they must not be underestimated, because they could be the cause of future problems in antimicrobial therapy.

The strain belonging to biotype 1, serotype O:6 was the only strain possessing pyrazinamidase activity. This strain was not found to harbor any plasmids. No other strain, including the biotype 4, serotype O:3 strain not harboring any plasmids, exhibited such an activity. It seems clear that the pyrazinamidase test is not correlated with the presence of plasmids.

Our data confirm the usefulness of this test in distinguishing between pathogenic and saprophytic strains (10); in fact, we found that none of the isolates with bioserotypes considered to be pathogenic for humans presented such an activity. Furthermore, we noted that all the strains which were

negative for the pyrazinamidase test also failed to hydrolyze esculin.

The correlation between plasmid content and some pathogenicity tests in vivo and in vitro showed that all strains harboring a DNA plasmid of 42 to 48 MDa gave the same positive responses to the calcium dependency test and the autoagglutination test and had an LD<sub>50</sub> lower than 10<sup>7</sup> CFU/ml. The only two strains without such plasmids were calcium independent and not agglutinable, and they had an LD<sub>50</sub> exceeding 10<sup>7</sup> CFU/ml.

As reported by other authors (20, 26), invasiveness in HEP-2 cells was not found to be plasmid dependent. In fact, the two strains not harboring plasmids showed different behavior: the biotype 1, serotype O:6 strain was not able to invade HEP-2 cells, whereas the biotype 4, serotype O:3 strain was able to do so.

*Y. enterocolitica* virulence is a complex phenomenon; it is, however, clear that the virulence plasmid plays an important role in contributing to or determining the virulence either as a sole factor or in cooperation with chromosomal determinants.

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