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Virulence and Phenotypic Characterization of *Yersinia enterocolitica* Isolated from Humans in the United States

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Yersinia enterocolitica was recently reclassified into *Yersinia enterocolitica* sensu stricto and three additional species. With this new classification, it was of interest to reexamine pathogenicity previously ascribed to *Y. enterocolitica*. All available clinical isolates of *Y. enterocolitica* sent to the Centers for Disease Control from 1970 through 1980 were selected for characterization and comparison. One-hundred such strains had been submitted, from 21 states. Most (85%) were biotype 1, and O:8 was the most common of the 24 serotypes encountered. All strains were examined by several virulence assays. Two strains caused conjunctivitis in guinea pigs, 7 were lethal for mice, 54 invaded HEp2 cells, 18 produced a heat-stable enterotoxin, 9 were calcium dependent, 20 autoagglutinated, and 34 had a distinctive colonial morphology at 37°C. Ten isolates of each of the new species that had previously been grouped with *Y. enterocolitica* (*Y. kristensenii*, *Y. intermedia*, and *Y. frederiksenii*) were characterized and were generally negative in all assays. This study points out pathogenicity differences among *Yersinia* species, confirms the complex nature of virulence in *Y. enterocolitica*, and confirms that no single current assay correlates with virulence in *Y. enterocolitica*.

A series of publications in 1980 defined the species *Yersinia enterocolitica* and proposed the creation of three new *Yersinia* species: *Y. intermedia*, *Y. kristensenii*, and *Y. frederiksenii* (3, 4, 8, 9, 28). These proposals were made after extensive studies of DNA relatedness and biochemical characteristics. The newly described species reflect the diversity of biochemical and epidemiological observations of the former atypical or *Y. enterocolitica*-like organisms. The more precise definition of *Y. enterocolitica* sensu stricto may clarify the diverse, and often perplexing, characteristics previously ascribed to this organism.

The clinical and epidemiological significance of the three newly described *Yersinia* species is uncertain at present. Past reports have principally associated strains of these three species with animal and environmental origins, and their relationships to human disease are unknown (5, 6, 26). Although these strains are usually considered to be nonpathogenic for humans, it has been (5) suggested that they may become host adapted for humans and be clinically significant in the future. Currently, however, *Y. enterocolitica* alone is recognized as the significant human pathogen of these four species (5).

In 1980, we initiated a study of all of the available (100) *Y. enterocolitica* sensu stricto strains isolated from humans in the United States and sent to the Centers For Disease Control (CDC) for identification or confirmation over the past 10 years. This study was undertaken in an attempt to characterize these strains by biotype, serotype, antibiotic susceptibility, and potential virulence. Representatives of each of the three new species were to be similarly characterized and compared with *Y. enterocolitica* strains. One of the primary goals of this research was to establish the potential pathogenicity of these *Y. enterocolitica* isolates by using well-documented assays that have been reported to be indicators of virulence (12, 15–17, 24). Our initial problem was to relate the results of virulence assays to virulence in humans.

In this investigation, we used epidemiologically incriminated strains of *Y. enterocolitica* as positive control cultures for the virulence assays (12, 14, 32). These strains were analyzed previously for genes governing their virulence for mice (12). Our avirulent controls were spontaneous derivatives of the virulent strains which no

TABLE 1. Source and geographical origin of 100 strains of *Y. enterocolitica*

CDC enteric number	Source of specimen	Origin of specimen	CDC enteric number	Source of specimen	Origin of specimen
503-70	Unknown	U.S.A.	1338-77	Unknown	Wisconsin
2138-72	Unknown	Georgia	1339-77	Unknown	Wisconsin
2139-72	Unknown	Georiga	1693-77	Stool	Louisiana
815-73	Unknown	Maryland	9300-78	Unknown	New York
905-74	Unknown	U.S.A.	9302-78	Stool	California
906-74	Unknown	U.S.A.	9303-78	Blood	New York
907-74	Unknown	U.S.A.	9304-78	Blood	New York
1223-75	Stool	North Carolina	9311-78	Stool	Georgia
1224-75	Stool	North Carolina	9312-78	Stool	Georgia
1529-75	Stool	Pennsylvania	9314-78	Spleen	Georgia
2164-75	Stool	California	9315-78	Blood	U.S.A.
2165-75	Stool	California	9316-78	Unknown	California
2338-75	Blood	New Jersey	9317-78	Unknown	New York
3968-76	Stool	Wisconsin	9319-78	Blood	New York
3971-76	Stool	Wisconsin	9322-78	Unknown	Kentucky
3972-76	Stool	Wsiconsin	9323-78	Unknown	Virginia
3973-76	Stool	Wisconsin	9324-78	Unknown	Alabama
3974-76	Stool	Wisconsin	9286-78	Stool	Kentucky
3975-76	Stool	Wisconsin	9291-78	Stool	Kentucky
3976-76	Stool	Wisconsin	9292-78T	Stool	Kentucky
1811-77	Unknown	Wisconsin	9294-78	Stool	Kentucky
1812-77	Unknown	Wisconsin	9295-78	Stool	Kentucky
2339-77	Blood	Connecticut	9297-78	Unknown	Florida
2648-77	Unknown	U.S.A.	9299-78	Wound	New York
2649-77	Unknown	Wisconsin	9369-78	Unknown	California
2650-77	Unknown	Wisconsin	9381-78	Unknown	California
67-78	Blood	Mississippi	9383-78	Unknown	New Jersey
200-78	Sputum	New Jersey	9385-78	Unknown	North Carolina
1746-78	Stool	New Hampshire	9387-78	Unknown	Maryland
2214-78	Human milk	Massachusetts	9394-78	Wound	North Carolina
9139-78	Unknown	U.S.A.	9395-78	Unknown	Washington
9155-78	Unknown	U.S.A.	9468-78	Stool	New York
9283-78	Stool	Michigan	9484-78	Stool	Connecticut
9284-78	Stool	Washington	256-79	Tissue	Texas
3977-76	Stool	Wisconsin	9326-78	Unknown	Louisiana
3978-76	Stool	Wisconsin	9327-78	Stool	North Carolina
3979-76	Stool	Wisconsin	9328-78	Abscess	Tennessee
183-77	Stool	Wisconsin	9332-78	Stool	Kentucky
184-77	Stool	Wisconsin	9333-78	Stool	Kentucky
185-77	Stool	Wisconsin	9334-78	Stool	Kentucky
453-77	Gall bladder	Connecticut	9366-78	Tissue	Georgia
678-77	Unknown	Wisconsin	962-79	Tissue	Texas
679-77	Unknown	Wisconsin	1209-79	Blood	North Carolina
681-77	Unknown	Wisconsin	2492-79	Stool	Michigan
744-77	Sputum	Connecticut	2519-79	Stool	New Hampshire
802-77	Stool	New Jersey	2570-79	Stool	Oregon
874-77	Stool	New Jersey	43-80	Sputum	New York
940-77	Stool	New Jersey	603-80	Stool	Massachusetts
1137-77	Lymph node	Pennsylvania	A2627	Stool	New York
1324-77	Sputum	Connecticut	A2628	Stool	New York

longer contained the implicated genes and which were no longer virulent for mice (12). A better or more accurate definition of virulence for humans might be achieved by feeding *Y. enterocolitica* strains to volunteer human subjects; however, we were obliged to accept the epidemiological and microbiological definition.

MATERIALS AND METHODS

The abbreviations used for tests described in this paper are: CM37, temperature-dependent colony morphology; AA, temperature-dependent autoagglutination; CAD, temperature-dependent calcium requirement; ST, heat-stable toxin production; TC, epithelial cell penetration; EYE, conjunctivitis in guinea pig eye;

TABLE 2. Phenotypic characteristics of 100 strains of *Y. enterocolitica*

CDC enteric number	Serotype	Biotype[a]	Antibiotic resistances[b]
503-70	O:8	1	SD,CF
2138-72	NT[c]	1	SD,CF,AM
2139-72	O:34	1	CB,AM
815-73	O:5,27	2	SD,CF
905-74	O:13,18	1	CB,AM
906-74	NTR[d]	1	SD,CF,AM
907-74	O:TACOMA[e] O:4	1	SXT,SD
1223-75	O:20	1	Susceptible
1224-75	O:7,8	1	CB
1529-75	O:5,27	1	CB,CF,AM
2164-75	O:7,8,13	1	CB,CF,AM
2165-75	O:7,8,13	1	CB,CF,AM
2338-75	O:5	NB[f]	CB,SD,CF,AM
3968-76	NTX[g]	1	CB,SD,CF,AM
3971-76	NTX	1	CB,CF,AM
3972-76	O:6	1	CB,CF,AM
3973-76	O:4	1	SD
3974-76	NTR	1	CB,CF,AM
3975-76	O:7,8	1	CB,SD,CF,AM
3976-76	NTR	2	CB,CF,AM
1811-77	NTR	1	CB,CF,AM
1812-77	O:5	1	CB,CF,AM
2339-77	O:5	2	SD,AM
2648-77	O:8	1	CB,CF,AM
2649-77	NTR	1	CB,CF,AM
2650-77	O:5	1	CB,CF,AM
67-78	O:5	2	CF
200-78	NT	1	CB,SD,CF,AM
1746-78	O:5	2	SD,CF,AM
2214-78	NTR	1	CB,AM
9139-78	O:7,8,13	1	Susceptible
9155-78	O:8	1	Susceptible
9283-78	O:8	1	Susceptible
3977-76	O:5	NB	CB,SXT,SD,CF,AM
3978-76	NTR	1	CB,CF,AM
3979-76	NTR	1	CB,CF,AM
183-77	O:8	1	CB,CF,AM
184-77	NTR	1	CB,CF,AM
185-77	NTR	1	CB,CF,AM
453-77	O:19	1	CB,CF,AM,S
678-77	O:6	1	CB,SD,CF,AM
679-77	O:6, O:28	1	CB,CF,AM
681-77	O:19, O:16	1	CB,CF,AM
744-77	O:8	1	CB,SD,CF,AM
802-77	O:8	1	CB,CF,AM
874-77	O:20	1	CB,CF,AM
940-77	O:6	1	CB,CF,AM
1137-77	O:8	1	SD
1324-77	NTR	1	SD,CF,AM
1338-77	O:6	1	CB,CF,AM
1339-77	O:6	1	CB,CF,AM
1693-77	O:11,23	1	CB,CF,AM
9300-78	O:8	1	CB,AM
9302-78	NTR	1	CB,SD,CF,AM
9303-78	O:18	1	CB,CF,AM
9304-78	O:18	1	CB
9311-78	O:13	NB	CB,CF,AM
9212-78	O:13,18	NB	CB,SD,CF,AM
9314-78	O:5,27	3	CB,SD,CF,AM
9315-78	O:8	1	SD,CF
9316-78	O:8	1	CF
9317-78	O:8	1	SD,CF

TABLE 2—*Continued*

CDC enteric number	Serotype	Biotype[a]	Antibiotic resistances[b]
9319-78	O:8	1	CB,SD,CF,AM
9322-78	O:20	1	CF
9323-78	O:13,18	1	CB,CF,AM
9284-78	O:8	NB	SD,CF,AM
9286-78	O:20	1	CB
9291-78	O:6,30	1	CB,CF,AM
9292-78T	O:6,31	2	CF,AM
9294-78	NTR	1	CB,CF
9295-78	O:18	1	CB,AM
9297-78	O:21	1	CB,SD,CF,AM
9299-78	O:8	1	CB
9369-78	O:6,31	1	CB,CF,AM
9381-78	NT	1	CB,CF,AM
9383-78	O:20	1	CB,SD,CF,AM
9385-78	O:18	1	CB,CF,AM
9387-78	O:14	1	CB,CF,AM
9394-78	O:6,31	1	CB,CF,AM
9395-78	O:8	1	CF,AM
9468-78	O:TACOMA, O:40	1	CB,SD,CF,AM
9484-78	O:5	2	SD,CF,AM
256-79	O:18	1	CB,SD,CF,AM
9324-78	O:8	1	CF
9326-78	O:8	1	SD,CF,AM
9327-78	O:20	1	CB,CF
9328-78	O:8	1	SD,CF
9332-78	O:11,23	1	CB,CF,AM
9333-78	O:19	NB	CB,SD,CF,AM
9334-78	O:11,13	1	CB,CF,AM
9366-78	O:8	1	CB,SD,CF
962-79	O:8	1	CB,CF
1209-79	O:13	1	CB,SD,CF,CL,AM
2492-79	O:7,13	1	CB,CF,AM
2519-79	O:3	NB	CB,SD,CF,AM
2570-79	O:20	1	CB,CF,AM
43-80	O:20	1	CB,SXT,SD,CF,AM
603-80	O:6	1	CB,SD,CF,AM
A2627	O:8	1	CF
A2628	O:8	1	CF

[a] Biotyping scheme of Bercovier et al. (3).
[b] CF, Cephalothin; AM, ampicillin; CB, carbenicillin; SD, sulfadiazine; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; CL, colistin.
[c] NT, Not typable, nonreactive.
[d] NTR, Not typable, rough.
[e] New unnumbered serotype.
[f] NB, Not biotypable by the scheme of Bercovier et al.
[g] NTX, Not typable, multiple cross-reactions.

and LETH, mouse lethality by intraperitoneal inoculation. Positive and negative test results are indicated by a + or −, respectively, after the test abbreviation.

Study strains. Human strains of *Y. enterocolitica* isolated in the United States were selected as a study population. Isolates were obtained from the Enterobacteriology Section stock collection and had been received by CDC between 1970 and 1980. A total of 100 strains met the project criteria (viz., *Y. enterocolitica* of human origin from persons in the United States). These strains along with their sources and geographical origins are listed in Table 1.

Virulence control strains. Three previously described, outbreak-associated strains of *Y. enterocolitica* were chosen as virulent control cultures: (i) strain WA, originally isolated from a patient with septicemia (14); (ii) strain A2635 recovered from chocolate milk that caused a foodborne outbreak of gastroenteritis (32); and (iii) strain Y7P, likewise recovered from a patient with gastroenteritis (12). Spontaneously occurring, avirulent (as measured in three assays for virulence) (12) derivatives of each of these strains were chosen as negative control cultures.

***Yersinia* species controls.** Ten each of the newly described *Yersinia* species were selected as species controls of unknown virulence. These strains originat-

ed from a variety of sources and locations and are generally considered to be nonpathogenic, or at least not associated with disease typical of that caused by *Y. enterocolitica* (4, 9, 28).

Culture storage. All study and control cultures were stocked at room temperature on blood agar base slants with paraffin-coated corks before June 1981. During 1981 and 1982, all study and control strains were subcultured and also stocked at $-70°C$ in 50% glycerol–50% brain heart infusion broth.

Biotype. All *Yersinia* strains were biochemically characterized, and the *Y. enterocolitica* strains were assigned to one of the five biotypes defined by Bercovier et al. (3). The API 20E Enteric Identification system (Analytab Products, Plainview, N.Y.) was used for biochemical identification. Tests were done according to the manufacturer's instructions, and the cultures were incubated at 25°C.

Serotype. Serotyping was done by a modification of the Widal whole cell agglutination method (30). The modification and a description of various antigens and antisera have been published elsewhere (21). Reference cultures to confirm the specificity of antisera were obtained for serotypes O:1 through O:23 and O:25 through O:34 from H. Bercovier, Pasteur Institute, Paris, France.

Antibiotic susceptibilities. Antimicrobial susceptibility tests were performed according to standard procedures (1, 2, 10). Antibiotics were chosen on the basis of their clinical, epidemiological, and genetic appropriateness. Inoculated plates were incubated for 18 to 24 h at 25°C. Zones of bacterial inhibition were measured to the nearest millimeter and recorded.

CM37. A unique colonial morphology designated CM37+, was used to select *Y. enterocolitica* colonies associated with virulence for mice (J. Lazere and P. Gemski, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B11, p. 19). All study and control strains were streaked for isolated colonies on tryptic soy agar plates. The plates were then incubated at 37°C for 18 to 24 h. After incubation, plates were examined with a binocular dissecting microscope, using obliquely transmitted light. Small, smooth, opaque colonies with a somewhat raised crown were isolated as presumptive virulent clones (CM37+). Presumptively avirulent colonies were larger, flatter, and more translucent.

AA. AA of *Y. enterocolitica* has been reported to be associated with bacterial virulence in mice (15). Methods are described elsewhere (15).

CAD. Magnesium oxalate agar was prepared as described elsewhere (12). Test strains of *Yersinia* were grown in brain heart infusion broth overnight at 25°C and diluted with sterile saline (0.85% NaCl) to a concentration of approximately 10^4 cells per ml. Duplicate samples containing 10 μl of the diluted bacterial suspension were spread over the entire surface of two magnesium oxalate agar plates. Both plates were incubated for 48 h, one plate at 25°C and one plate at 37°C. Growth on the two plates was compared. The 25°C plate served as a growth standard (uninhibited). Inhibition of bacterial growth at 37°C (fewer numbers or markedly smaller colonies) was evidence of a positive test (CAD+). Equal numbers of similarly sized colonies on magnesium oxalate agar plates at both temperatures were indicative of a negative test (CAD−).

Toxin production. The production of a heat-stable toxin by study strains was determined by the infant mouse assay (11). Strains were grown at 25°C for 48 h with aeration in 3 ml of tryptic soy broth with 0.6% yeast extract. Filtrates containing Evans blue dye were injected intragastrically in 2- to 4-day-old mice. After 2.5 h of incubation at room temperature, mice were sacrificed, and a ratio of the intestinal weight to the remaining body weight was determined. A ratio of greater than 0.083 was considered ST+ (7, 13). The enzyme-linked immunosorbent assay procedure described by Yolken et al. (31) was used to assay for the production of labile toxin.

HEp-2 penetration and monolayer detachment. HEp-2 was maintained by the CDC Tissue Culture Laboratory. The following modifications were made of previously described assays of TC (16, 20, 27). Tissue culture flasks (25 cm^2; Corning Glass Works, Corning, N.Y.) were seeded with approximately 1.5×10^5 *Mycoplasma*-free HEp-2 cells and grown to near confluency. Before bacterial inoculation, monolayers were washed three times with phosphate-buffered saline, pH 7.4. A broth culture of approximately 10^8 cells was prepared by inoculating 5 ml of tryptic soy broth and incubating at 25°C for 18 h. After incubation, cells were pelleted and then suspended in 2 ml of Dulbecco modified minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) with 2% fetal calf serum. This inoculum was placed on the washed monolayer and allowed to remain for 90 min at room temperature. Monolayers were then washed three times with phosphate-buffered saline fixed in absolute methanol, stained for 15 min with undiluted Giemsa stain, and observed microscopically. Two parameters were examined: the ability of strains to penetrate HEp-2 cells and the ability of strains to cause toxic detachment of HEp-2 monolayers. The monolayer detachment assay was performed as above with modifications described elsewhere (20).

Electron microscopy. Electron microscopic examination of HEp-2 cells incubated with *Y. enterocolitica* was accomplished by fixing the monolayers in situ with 2% (vol/vol) glutaraldehyde in 0.1 M *S*-collidine buffer for 1 h and then rinsing with 0.1 M *S*-collidine buffer. Cells were subsequently removed from the flask, centrifuged, and pelleted. After postfixation for 45 min in 1% (vol/vol) OsO_4 in *S*-collidine buffer, pellets were stained in a saturated solution of uranyl acetate in 70% ethanol for 3 h, dehydrated through graded concentrations of ethanol, and embedded in resin. Thin sections were cut, mounted on bare copper grids, and examined with a Philips 200 electron microscope (Philips Electronic Instruments, Mt. Vernon, N.Y.) at 40 kV accelerating voltage.

EYE. Due to the expense and support systems necessary for guinea pig maintenance, we selected 46 *Y. enterocolitica* strains for testing in experimental animals (24, 32). All strains giving positive reactions in any other assay were chosen, and all strains of serotype O:8 were chosen because this serotype has been closely associated with invasive human disease in the United States (14, 20, 32). Additionally, two representatives of each *Yersinia* species control were tested (total of six). Therefore, 52 cultures were assayed.

Tryptic soy agar plates were inoculated for maximum growth by swabbing with an overnight broth culture (brain heart infusion broth, 25°C) of the strain to be tested. After incubation at 25°C for 48 h, growth from the tryptic soy agar plates was harvested and

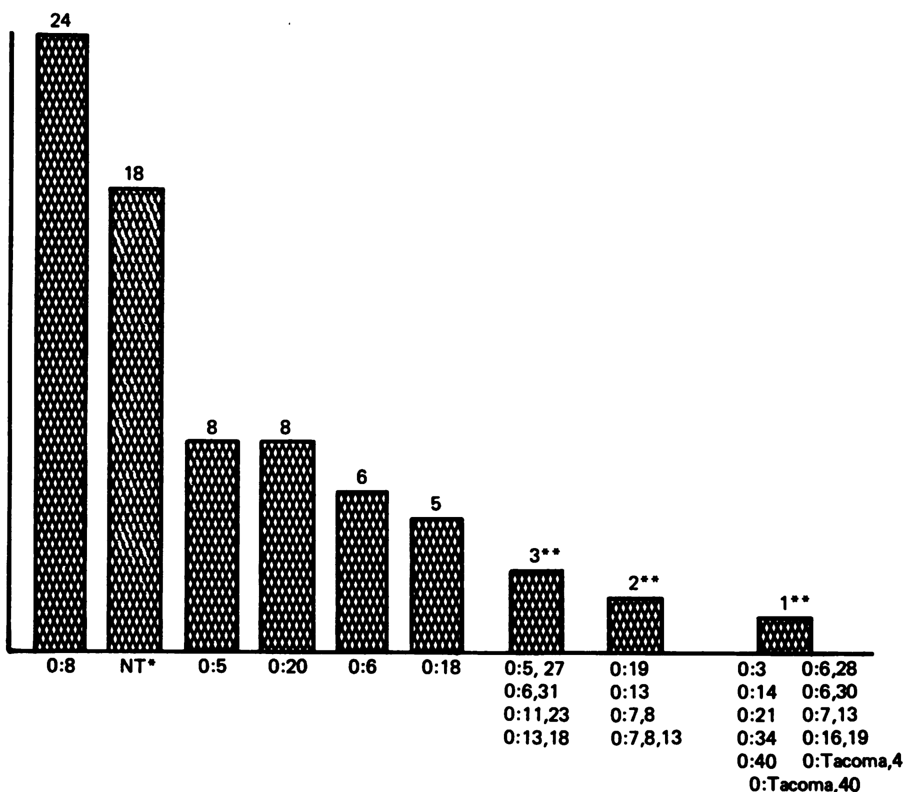

FIG. 1. Serotypes of 100 strains of *Y. enterocolitica*. NT*, Not typable; **, per serotype.

emulsified in 2 to 3 drops of sterile saline. One drop was introduced into the right eye of an adult female guinea pig (CDC Animal Facility, Lawrenceville, Ga.). Animals were examined daily for a period of up to 7 days for evidence of eye infection characterized by swelling, eyeball depression, accumulation of fluid, mucous, blood, or corneal opacity. Failure to develop evidence of infection within 7 days was regarded as a negative test (EYE−). Transient irritation (redness, lacrimation) was not considered significant.

LETH. Pairs of adult ICR mice were inoculated intraperitoneally with approximately 5×10^6 bacterial cells in 0.5 ml. The inoculum was prepared by growing strains in 5 ml of brain heart infusion broth at 25°C for 18 h, pelleting cells, and suspending in sterile saline to the turbidity of a 0.5 McFarland standard (1). The inoculum was confirmed by agar plate counts. After intraperitoneal inoculation, mice were observed daily for up to 21 days for signs of illness or death. Deaths occurring within 24 h of inoculation were attributed to endotoxic shock, in which case a second pair of mice was inoculated.

RESULTS

Biotyping results are presented in Table 2. As shown, 85 strains (85%) were of biotype 1, 7% were of biotype 2, and 1 strain (1%) was of biotype 3. No strains were biotype 4 or 5. Of the 100 strains examined, 7 were not biotypable by the scheme of Bercovier et al. (3).

The *Y. enterocolitica* study strains contained 24 serotypes. These results are summarized in Table 2 and Fig. 1. Serotype O:8 occurred most frequently (24%) followed by serotypes O:5 (8%), O:20 (8%), O:6 (6%), and O:18 (5%). Eighteen strains were not typable. The remaining strains were distributed among 19 serotypic patterns with low frequency.

Antimicrobial resistances for *Y. enterocolitica* strains are given in Table 2. Most strains of all four species were resistant to cephalothin, ampicillin, and carbenicillin. With the exception of *Y. frederiksenii*, all strains showed some degree of resistance to sulfadiazine: *Y. enterocolitica*, 37%; *Y. kristensenii*, 20%; and *Y. intermedia*, 10%. *Y. enterocolitica* alone showed resistance to trimethoprim-sulfamethoxazole (3%), colistin (1%), and streptomycin (1%). One strain of *Y. intermedia* was resistant to chloramphenicol (10%).

The experimental results of all virulence assays for *Y. enterocolitica* control strains are presented in Table 3, and those for study strains are presented in Table 4. Data for *Yersinia* species control strains are presented in Table 5. A total of 34 *Y. enterocolitica* strains representing 12 serotypes were CM37+. Approximately 50% of colonies from CM37+ strains had the

TABLE 3. Results of seven assays for virulence-associated characteristics of *Y. enterocolitica* control strains

Strain	Test						
	CM37	AA	CAD	ST	TC	EYE	LETH
WA+	+	+	+	+	+	+	+
WA−[a]	−	−	−	+	+	−	−
A2635	+	+	+	−	+	+	+
TAMU-75[b]	−	−	−	−	+	−	−

[a] Cured derivative of WA+.
[b] Cured derivative of A2635.

virulence-associated morphology. In CM37− cultures, no colony variations were observed. All virulent controls were CM37+, and all avirulent controls were CM37− (Table 3). Strains of the other *Yersinia* species were uniformly negative (Table 5).

Overall, 20 strains of *Y. enterocolitica* representing six serotypes (Table 4) were AA+. Virulent control strains were positive, and avirulent control strains were negative. The other *Yersinia* species were uniformly AA−.

Results of the CAD assay are also presented in Table 4. Nine strains representing nine serotypes of *Y. enterocolitica* were CAD+ by this test. All virulent controls were positive, and avirulent controls were negative. No strains of the other *Yersinia* species were CAD+.

Eighteen *Y. enterocolitica* study strains representing nine different serotypes were ST+ (Table 4). One virulent control strain was positive; its avirulent derivative was also ST+ (Table 3). Additionally, seven strains (23%) of the other *Yersinia* species were ST+. Five of these seven were strains of *Y. kristensenii*, and the remaining two strains were one strain each of *Y. intermedia* and *Y. frederiksenii* (see Table 5). All strains of the four *Yersinia* species tested were negative for labile toxin production as measured by enzyme-linked immunosorbent assay.

A total of 54 *Y. enterocolitica* strains representing 14 serotypes were positive for HEp-2 cell penetration (TC+) (Table 4). Virulent controls were TC+, but avirulent controls were also TC+ (Table 3). Two strains of *Y. intermedia* were TC+; no strain of *Y. frederiksenii* or *Y. kristensenii* was positive in this assay. The penetration results obtained with light microscopy were confirmed by electron microscopy, which revealed intracellular bacteria with TC+ *Yersinia* strains. The monolayer detachment assay was negative with both study and virulence control strains.

LETH determination showed that seven strains representing four serotypes were LETH+ (Table 4). All virulent control strains killed mice, and no avirulent derivative killed mice (by definition). No environment or *Yersinia* species control strain killed mice.

Two study and both virulence control strains of *Y. enterocolitica* were EYE+; all of these strains were serotype O:8. None of the other *Yersinia* species were EYE+.

DISCUSSION

As stated above, the *Y. enterocolitica* strains of known human virulence isolated in the United States are those virulent control cultures presented in Table 3. There is an unavoidable circularity involved in selecting avirulent derivatives of these by an assay (LETH) rather than by using human volunteers. Absence of mouse lethality, in our analysis, is given greater weight than all other assays postulated as measures of yersinial virulence for humans (12, 13, 15, 17, 19–21, 23, 24). There was no strain of *Y. enterocolitica* or other *Yersinia* species which was comparable in all assays to the virulence control cultures, i.e., CM37+, AA+, CAD+, ST+/−, TC+, EYE+, LETH+. As a result, we considered each virulence assay separately.

In the past, serotyping of *Y. enterocolitica* has been the primary tool for the identification of those clinically and epidemiologically significant strains (21). Although the recent definition of *Y. enterocolitica* sensu stricto has eliminated at least 14 of the 53 previously described serofactors, recent clinical references fail to reflect this (22, 29). Additionally, our experience and data suggest that the numerous and high-titer cross-reactions (indicating multiple serofactors per strain in many circumstances) and lack of reactions (18%) make interpretation difficult. Serotype does appear to be a useful strain characteristic for clinical and epidemiological purposes, but it is not easily adapted to the routine laboratory and may not always predict virulence. Examples of this are the eight serotype O:8 strains which do not invade the guinea pig eye, the two which do, and the two serotype O:8 strains which are unreactive in any of the virulence-associated assays. There are also five strains containing the serofactor O:8 plus other factors, and these give equally ambiguous results, although none invade the eye tissues.

Although serotype O:9 is the second most common human serotype on a global basis (e.g., Europe, South Africa, Japan, and Canada), none of our U.S. study strains were O:9 (25). Similarly, only one strain was identified as serotype O:3, which is the most common cause of disease in neighboring Canada and in much of Europe (25). This occurrence of specific serotypes in a particular, well-defined geographical area seems to be a characteristic of *Y. enterocolitica* in this study, and as previously reported.

Before our study, the CM37 procedure had been used exclusively with *Y. enterocolitica*

TABLE 4. Results of seven assays for virulence-associated characteristics of 100 strains of *Y. enterocolitica*

Strain identification	Test[a]						
	CM37	AA	CAD	ST	TC	EYE[b]	LETH
503-70				+	+	−	
2138-72		+		+	+	−	
2139-72			+		+		
815-73	+	+	+	+	+	−	
905-74							
906-74		+			+	−	
907-74					+		
1223-75	+	+	+	+	+	−	+
1224-75	+				+	−	
1529-75	+						
2164-75							
2165-75							
2338-75					+		
3968-76							
3971-76							
3972-76							
3973-76	+	+	+		+	−	+
3974-76							
3975-76					+		
3976-76		+				−	
3977-76				+	+	−	
3978-76							
3979-76							
183-77						−	
184-77							
185-77	+						
453-77							
678-77							
679-77				+		−	
681-77				+		−	
744-77					+	−	
802-77					+	−	
874-77	+	+			+	−	+
940-77							
1137-77	+	+	+		+	−	+
1324-77						−	
1338-77							
1339-77	+						
1693-77							
1811-77							
1812-77				+		−	
2339-77							
2648-77						−	
2649-77							
2650-77							
67-78	+				+		
200-78	+						
1746-78	+		+		+		
2214-78							
9139-78	+				+	−	
9155-78	+				+	−	
9283-78	+			+	+	−	
9284-78					+	−	
9286-78					+		
9291-78							
9292-78T			+				
9294-78	+						
9295-78				+	+	−	
9297-78	+			+	+	−	
9299-78		+			+	−	
9300-78		+			+	−	
9302-78		+	+	+	+	−	
9303-78					+		
9304-78					+		
9311-78	+	+			+	−	
9312-78	+				+		
9314-78					+		
9315-78	+	+			+	−	
9316-78	+				+	−	
9317-78	+			+	+	−	
9319-78	+	+				−	
9322-78					+		
9323-78				+	+	−	
9324-78					+	−	
9326-78					+	−	
9327-78					+		
9328-78				+	+	−	
9332-78							
9333-78	+	+			+	−	
9334-78							
9366-78	+			+	+	−	
9369-78							
9381-78							
9383-78	+	+			+	−	
9385-78							
9387-78							
9394-78						−	
9395-78	+	+			+	−	
9468-78					+		
9484-78	+				+		
256-79	+				+		
962-79					+	−	
1209-79	+	+	+		+	−	+
2492-79							
2519-79					+		
2570-79	+						
43-80							
603-80	+						
A2627	+	+		+	+	+	+
A2628	+	+		+	+	+	+

[a] Only positive reactions are listed for assays other than EYE; all strains were tested in each assay other than EYE.

[b] Only 46 of the 100 strains were tested in EYE (see the text).

serotypes O:3 and O:8. Our data indicate that no fewer than 12 serotypes have CM37+ strains, suggesting that the colony morphology associated with mouse virulence may be widespread within *Y. enterocolitica*, but is not present in the newly designated *Yersinia* species. This association with virulence is strengthened by the occurrence of CM37-avirulent control cultures.

Laird and Cavanaugh (15) first described the AA phenomenon and reported that 25 of 180 *Y.*

TABLE 5. Results of seven assays for virulence-associated characteristics of 30 *Yersinia* (not *Y. enterocolitica*) control strains

Species	No. of isolates positive for:						
	CM37	AA	CAD	ST	TC	EYE[a]	LETH
Y. frederiksenii[b]	0	0	0	1	0	0	0
Y. kristensenii[b]	0	0	0	5	0	0	0
Y. intermedia[b]	0	0	0	1	2	0	0

[a] Six strains, two from each species.
[b] $n = 10$.

enterocolitica-complex (before the 1980 taxonomic division) strains were AA+. All 25 of their AA+ strains were reported to be virulent for mice by the oral route. None of their AA− strains were virulent by this method. They felt that this test enabled them to accurately distinguish virulent and avirulent strains of *Yersinia*. Our avirulent controls were accordingly AA−. Selecting by colonial morphology at 37°C and testing by AA reaction revealed that 14 *Y. enterocolitica* strains were positive by both assays. Nineteen CM37+ strains were AA−, and six AA+ strains were CM37−. Although both assays have been reported as measures of mouse virulence, only 7 of our 14 CM37+, AA+ strains were lethal for mice. All LETH+ strains were CM37+ and AA+.

AA+ reactions have been reported for strains of the presumptive pathogenic serotypes O:3, O:8, and O:9 and, more recently, for strains of serotypes O:5,27, O:4,32, and O:21 (23). Our data suggest four previously unreported AA+ *Y. enterocolitica* strains of serotype O:20, O:13, O:19, and O:40. Many of these serotypes are poorly, or not at all, associated with human illness. One of the most interesting aspects of these data is that all of the 30 *Yersinia* species control strains were both CM37− and AA−.

Only nine of the *Y. enterocolitica* strains expressed calcium dependency, and these strains were generally positive in other assays; each strain represented a different serotype. Despite the fact that virulence control strains and other O:8 study strains gave very clearly defined reactions as described in the literature for these and O:3 serotype strains (12) and all strains of other *Yersinia* species were clearly CAD−, our findings indicate that use of this test as a screen for virulence may have limitations. For example, one strain, 9292-78, was CAD+ and negative for all other assays of virulence. In addition, some of these data from our isolates were equivocal, requiring subjective interpretation of growth that occurred on magnesium oxalate agar plates at 37°C.

Although there have been published reports that the majority of human isolates of *Y. enterocolitica* are ST+ (16–18), the clinical presentation of yersiniosis and our data indicate that a minority of human clinical isolates of *Y. enterocolitica* are ST+. One virulent control culture was ST−, and although one virulent control culture was ST+, the avirulent derivative was also ST+. Additionally, our "implied avirulent" group of *Yersinia* species control strains contained a higher percentage of ST+ than the clinical isolates. In general, these data do not support the previously observed association of ST+ strains with human, rather than environmental, isolates of yersiniae.

As mentioned above, there is no clinical indication of enterotoxin production in yersiniosis. The labile toxin data confirm this. Labile toxin production has not been reported for yersiniae in the past, but has been found for *Vibrio cholerae*, *V. mimicus*, *Aeromonas*, *Escherichia coli*, *Citrobacter diversus*, and other enteric organisms (22). Other investigators have probably found similar and uniformly negative results for yersiniae, which were not submitted for publication.

The use of tissue culture cell penetration as an experimental model of in vivo invasive disease is consistent with the usual illness produced by *Y. enterocolitica*. However, epithelioid cells in culture may be more susceptible to penetration than intact tissues in vivo, or penetration may not reflect true invasion. Accordingly, Formalin- or UV-killed *Yersinia* have been reported to penetrate (internalize) HeLa cells (19), and the avirulent controls (Table 5) in this study remained TC+ (19). Conversely, *Yersinia* species control strains were significantly (93%) TC−. We speculate that *Yersinia* strains which are unable to penetrate cells in culture are probably not invasive for intact human tissues. The TC assay, which is simple and rapid to perform, may measure an incomplete but prerequisite characteristic of virulence. Results in almost all cases were unequivocal.

The tissue culture detachment assay was unsatisfactory in our hands; one strain which was reported positive by another laboratory using this procedure (20) gave negative results with our HEp2 cell line. Neither of our pathogenic control strains caused monolayer detachment after repeated examinations. Although promising in other laboratories, these uniformly negative results with known TC+ control strains discouraged our use of this assay with other study strains.

An animal model assaying for the actual invasion of tissues with subsequent in vivo systemic disease may best reflect the clinical presentation associated with human yersiniosis (ergo, yersinial virulence?). For this reason, we tested all study and control strains for mouse lethality. By definition, virulence control strains were

LETH+, and avirulent derivatives as well as *Yersinia* species control strains were LETH−. The seven LETH+ study strains were CM37+, AA+, and TC+. These data indicate that the LETH characteristic may require or depend on the presence of other strain characteristics such as cell wall properties (AA) and the ability to colonize or penetrate host epithelial cells or both (TC). Two of the seven LETH+ strains were EYE+; these were both serotype O:8. However, no other LETH+ or O:8 strain was EYE+. This was true for virulence control cultures also. We can say that, in our study, having the O:8 serofactor and being LETH+ appear to be necessary for EYE+ strains, but that relationship is not reciprocal.

Using the three United States *Y. enterocolitica* strains obtained during outbreaks of human illness as a standard against which to measure virulence, no study strain is fully virulent. We propose that this is not so much a reflection of the study population as it is of the positive control population. More outbreak-associated strains are needed, especially strains of serotypes known to cause disease in other countries (23, 25). Based on data from this study and conclusions drawn by other investigators (12, 23), we feel that the LETH assay best predicts yersinial virulence as measured in other assays and that its invasive disease may partially reflect human disease. It is less costly and less variable than most animal models and has an unequivocal endpoint.

Our data confirm the uniqueness of *Y. enterocolitica* sensu strictu, but indicate that diversity remains within the species, especially regarding the strain characteristics studied here. It is certainly not possible to say that all strains of *Y. enterocolitica* are pathogenic for humans and that *Y. intermedia, Y. kristensenii*, and *Y. frederiksenii* are nonpathogenic.

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