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Virulence of *Yersinia enterocolitica* Determined by Lethality in Mongolian Gerbils and by the Serény Test

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Virulence of human and environmental strains of *Yersinia enterocolitica* was evaluated by lethality in Mongolian gerbils and by the Serény test. The inducement of conjunctivitis in guinea pigs and lethality in gerbils after intraperitoneal administration was restricted to a few serotypes of *Y. enterocolitica*. Human strains of serotype O:8 were the most virulent, whereas strains of the common human serotype O:3 and most others were essentially avirulent. One virulent strain of serotype O:8 produced fatal infections within 7 days in four of five gerbils receiving 100 cells intraperitoneally. There was no observable difference in virulence between intraperitoneal and oral routes of administration, and upon death the infecting strain was found in high numbers in heart blood, lung, liver, and spleen. Virulence in both guinea pigs and gerbils was related to the presence of VW antigen determined by a nutritional requirement for calcium.

The first efforts to find a laboratory animal which could serve as a model for evaluating the pathogenicity of *Yersinia enterocolitica* were generally unsuccessful. Mollaret and Guillon (12) exposed 15 different animal species to 106 strains of *Y. enterocolitica* by various routes of administration and found none pathogenic for any animal. Strains of serotypes O:3 and O:9 isolated in Europe showed no pathogenicity for rabbits, guinea pigs, rats, or mice. Subsequently, Carter et al. (6), Carter and Collins (5), and then Quan et al. (16) demonstrated pathogenicity of strains of *Y. enterocolitica* isolated in the United States for laboratory mice. A number of other investigators have used laboratory mice to study the pathogenicity of *Y. enterocolitica* with various degrees of success (1, 11, 17). Maruyama (10) reported infections with pathological and serological responses in monkeys exposed to a human strain of serotype O:3. Une (21) reported experimental infections in rabbits after infection with strains of serotypes O:3 and O:9, but serotype O:5A and nontypable strains were avirulent. Wetzler et al. (22) first reported a lethal response in Mongolian gerbils (*Meriones unguiculatus*) to a strain of *Y. enterocolitica* with a 50% lethal dose of 500 cells administered intraperitoneally (i.p.). Quan et al. (16) challenged gerbils i.p. with a human strain of serotype O:8 and observed death within 4 days with as few as 250 cells. These investigators suggested that gerbils might serve as sensitive test animals for studying pathogenicity of *Y. enterocolitica*.

Virulence in *Yersinia pestis* (*Pasteurella pestis*) and *Yersinia pseudotuberculosis* (*Pasteurella pseudotuberculosis*) has been related to the presence of two antigens designated V and W (3, 4). Higuchi et al. (8) found that virulent types of *Y. pestis* have a calcium requirement for growth, whereas avirulent types will grow at 37°C on a calcium-deficient medium. Higuchi and Smith (9) subsequently developed a differential plating medium for detection of avirulent cell types of *Y. pestis*. Brubaker (2) reported that virulent strains of *Y. pseudotuberculosis* also have a nutritional requirement for calcium.

This paper reports the results of studies undertaken of two different animal systems for determining the virulence of *Y. enterocolitica*: (i) the Serény test with guinea pigs and (ii) lethality in gerbils. Since *Y. enterocolitica* is generically related to *Y. pestis* and *Y. pseudotuberculosis*, we added to our studies an examination for the presence of the VW antigen by ability to grow on a calcium-deficient medium and its relationship to virulence in the gerbil and Serény tests.

MATERIALS AND METHODS

***Y. enterocolitica* cultures.** The cultures of *Y. enterocolitica* used in these studies are listed in Table 1 along with their serotype, from whom they were obtained, and the original isolation source. All cultures were stored in deep stabs of tryptic soy agar (TSA) and were also frozen in mist dessicans at −70°C. Stock slants on TSA with 0.6% yeast extract (TSYE) were made from preserved cultures with one transfer to minimize the number of subcultures.

Culture number E572 is identified as a strain of serotype O:8, which is how it was identified when provided to us. We found that the strain was rough and untypable, and resisted all efforts to convert it to a smooth form. However, the organism was biochem-

TABLE 1. *Virulence of 41 Y. enterocolitica cultures in Mongolian gerbils*

Culture no.	Serotype O:	Obtained from	Isolated from	Dose (cells)	No. deaths/no. infected
E235	3	Our lab	Food	2.1×10^7	0/2
E546	3	Toma[a]	Human	2.5×10^7	0/2
E547	3	Toma	Human	9.0×10^6	0/2
E549	3	Toma	Human	4.4×10^6	0/3
E162	5	Our lab	Food	1.7×10^7	1/2
E417	5	Our lab	Food	6.0×10^6	0/3
E559	5	Toma	Human	1.8×10^7	1/1
				5.6×10^6	0/2
E560	5	Toma	Human	5.5×10^6	1/2
E132	8	Bissett[b]	Human	2.9×10^7	0/3
E213	8	Our lab	Food	6.1×10^6	0/3
E543	8	Toma	Human	2.6×10^7	2/2
E557	8	Toma	Human	7.6×10^6	3/3
E558	8	Toma	Human	2.0×10^7	3/3
E565	8	Wetzler[c]	Human	3.8×10^6	0/2
E572	8(R)	Wetzler	Human	9.5×10^6	2/2
E049	16	Christenson[d]	Human	1.3×10^7	0/3
E202	16	Our lab	Food	1.3×10^7	0/2
E517	18	Our lab	Water	6.5×10^6	0/3
E518	21	Our lab	Water	7.3×10^6	2/3
E561	4,32	Toma	Human	6.2×10^6	0/2
E562	4,32	Toma	Human	4.8×10^6	1/3
E554	4,33	Our lab	Food	9.7×10^6	0/3
E619	4,33	Our lab	Human	5.4×10^7	3/3
E083	5,27	Toma	Human	5.4×10^6	1/2
E544	5,27	Toma	Human	2.5×10^7	0/2
E555	5,27	Toma	Human	1.8×10^7	0/3
E556	5,27	Toma	Human	6.8×10^6	0/3
E545	6,30	Toma	Human	5.8×10^6	0/3
E612	6,30	Our lab	Human	5.0×10^6	0/2
E471	6,31	Our lab	Food	8.7×10^6	0/3
E616	6,31	Our lab	Human	1.1×10^7	0/3
E620	7,8	Our lab	Human	1.4×10^7	0/3
E621	7,8	Our lab	Human	8.8×10^6	1/3
E513	11,24	Our lab	Water	1.9×10^7	0/3
E119	13,7	Our lab	Food	1.0×10^7	0/2
E563	13,7	Toma	Human	1.2×10^7	0/3
				1.3×10^7	0/2
E564	13,7	Toma	Human	4.4×10^6	0/2
E526	16,29	Our lab	Human	4.5×10^6	0/3
E277	NT[e]	Our lab	Food	7.2×10^6	0/2
E626	NT	Toma	Human	5.5×10^6	0/3
E629	NT	Toma	Human	6.0×10^5	0/3

[a] S. Toma, Canadian National Reference Service for *Yersinia*, Toronto, Ontario.
[b] M. Bissett, California State Department of Health, Berkeley.
[c] T. Wetzler, University of Washington, Seattle.
[d] E. Christenson, State Laboratory of Hygiene, Madison, Wis.
[e] NT, Nontypable.

ically typical for serotype O:8 (e.g., indole positive); therefore, its original identification has been retained but qualified as O:8(R).

Virulence for the gerbil. The 41 test strains used for gerbil infection studies, representing 15 serotypes and three nontypable isolates (Table 1), were subcultured onto TSYE agar and incubated at 32°C overnight. A minimum of 10 colonies were touched with a loop to inoculate 5 ml of TSYE broth. The broth was incubated at room temperature (20 to 22°C) in a roller apparatus for 48 h. The cells were recovered by centrifugation and suspended and diluted in phosphate-buffered saline to a density of about 10^7 cells per ml. The density of this suspension was verified by colony count. Each animal received 0.5 ml of the cell suspension by i.p. injection. Cell suspensions of selected cultures for comparison of i.p. and oral routes of administration were prepared similarly. The oral dose consisted of 0.5 ml administered by an animal feeding tube (Popper & Sons, Inc., New York, N.Y.). All animals were held up to 15 days for observation.

Each gerbil that expired during the observation

period was autopsied, and heart blood was removed aseptically for inoculation of a MacConkey agar plate. In the study of i.p. and oral routes, pieces of lung, liver, spleen, plus feces and intestinal fluid if present, were also removed for culture. After incubation of inoculated MacConkey agar plates at room temperature for 48 h, the identity of the recovered organism was confirmed by colonial appearance, and, in the case of typable strains, by slide agglutination with specific antiserum. The density of the organism in the tissue specimens was expressed as the relative growth obtained on MacConkey agar streaked from a homogenate.

Virulence by Serény test. The 31 cultures used for studies in the Serény test, representing 11 serotypes and three nontypable isolates (see Table 5), were prepared as described for gerbils, except that five tubes with 5 ml of TSYE broth were inoculated with 0.1 ml of a suspension prepared with 10 colonies from the TSYE agar plate. After incubation these broth cultures were consolidated for centrifugation. Cells were suspended in 0.5 ml of 0.1% peptone buffer, the density was determined by colony count, and 10 μl of this suspension was introduced by an automatic pipette below the lower eyelid of the right eye of each test animal. The left eye of the animal served as a negative control. Animals were observed for up to 7 days.

Responses in the guinea pig eye were classified as follows: no response observed (−); a very weak response which was transitory and only low numbers of the infecting organism were recovered by swabbing the conjunctivae (±); a mild reaction which was progressive and persisted with recovery of a significant number of bacteria by swabbing (+); and a strong reaction with copious exudate which persisted and with recovery of high numbers of bacteria by swabbing (++).

The organism was recovered from infected eyes with a swab used to inoculate a MacConkey agar plate. Recovery of the infecting strain was confirmed by colonial appearance after incubation at room temperature for 48 h, and, when typable strains were used, by slide agglutination with specific antiserum.

Determination of VW antigen. The presence of VW antigen in 12 cultures was determined with the calcium-deficient differential plating medium described by Higuchi and Smith (9). This medium, designated magnesium oxalate agar (MOX), was prepared by adding 2.68 g of sodium oxalate and 4.067 g of magnesium chloride·$6H_2O$ to 1 liter of TSA. The final pH was adjusted to 7.6.

Test cultures were grown on blood agar at room temperature for 48 h. Two or three suspensions of individual colonies were made in phosphate-buffered saline, and an appropriate dilution was spread onto duplicate plates of TSA and MOX to provide isolated colonies after incubation at 35°C for 20 to 24 h. Growth on TSA with no growth on MOX was taken as evidence that the particular colony selected from the blood agar plate represented cells bearing the VW antigen.

Cultures selected for animal inoculation were prepared by placing the TSA plate from 35°C incubation at room temperature for an additional 24 h. Growth was then washed off with phosphate-buffered saline, and 10 μl of this suspension was used to inoculate the eye of a guinea pig. A 10^{-3} dilution of the same suspension was made, and 0.5 ml was used to inject gerbils i.p. The density of the suspension was determined by colony count.

RESULTS

Virulence for gerbils. Table 1 shows the results obtained with infection of gerbils by the i.p. route for 41 strains of *Y. enterocolitica*. The most virulent strains were all of serotype O:8, with four of seven strains causing fatal infections in all of the animals exposed. One of two test strains of serotype O:4,33 caused fatal infections in three of three animals, whereas other strains representing serotypes O:5,27, O:5, O:21, O:4,32, and O:7,8 produced deaths in some but not all of the animals infected. Common serotypes isolated from humans in Canada, O:3, O:6,30, and O:5,27 (20), were essentially avirulent by the gerbil test.

One strain of serotype O:8 (E557) was titrated in gerbils (Table 2). The high fatality rate (i.e., four deaths out of five infected) with administration of only 100 cells indicates that this serotype is extremely virulent for gerbils.

Four strains of *Y. enterocolitica* were used to compare virulence by i.p. and oral routes of administration (Table 3). There was no observable difference in virulence with either i.p. or oral administration. The distribution of the organism upon autopsy (Table 4) suggests a similar invasive process after infection by i.p. and oral routes. However, there were some pathological differences between the animals observed upon autopsy. Those which had been infected i.p. tended to be devoid of fecal pellets and showed large accumulations of fluid in the stomach and small intestine. Animals infected orally did not show these effects but did have lung and thoracic hemorrhaging and frequently showed nasal bleeding immediately after death.

Virulence by Serény test. The results of the Serény test for 31 strains of *Y. enterocolitica* are presented in Table 5. In no case with a positive response did the infection involve the

TABLE 2. *Dose response of gerbils to Y. enterocolitica serotype O:8[a] administered by i.p. route*

No. of cells	Time (days) to death of animal no.:					Total deaths/ total infected
	1	2	3	4	5	
100,000	3	3	3	5	No animal	4/4
10,000	4	4	5	7	Lived	4/5
1,000	4	5	Lived	Lived	Lived	2/5
100	5	6	7	7	Lived	4/5

[a] Culture no. E557 (see Table 1).

cornea of the eye as is observed in classic keratoconjunctivitis produced by invasive shigellae (19). The iris was always visible and the pupil remained clear. The infection was limited to the conjunctivae only, and was characterized by a swelling and inflammation with depression of the eyeball and accumulation of a purulent exudate.

Of the 31 strains tested, 12 gave observable reactions in the guinea pig eye, which we shall call guinea pig conjunctivitis. These 12 strains represented only four serotypes, O:5,27, O:8, O:6,30, and O:3. Since only one of three animals showed a weak reaction (±) with one strain of O:6,30, and the second strain of this serotype gave no reaction in two animals exposed, this type of *Y. enterocolitica* appeared to be avirulent by this test procedure. In contrast, each of

TABLE 3. *Virulence of Y. enterocolitica for gerbils by oral and i.p. routes of administration*

Culture no.	Serotype	Dose (cells)	Animal no.	Time (days) to death[a]	
				i.p.	Oral
E547	O:3	1.8×10^7	1	12[b]	Lived
			2	Lived	Lived
E557	O:8	9.6×10^6	1	4	4
			2	2	3
E572	O:8(R)	2.3×10^7	1	2	3
			2	2	4
E619	O:4,33	8.6×10^6	1	Lived	Lived
			2	Lived	Lived

[a] Held 15 days for observation.
[b] Small, debilitated animal; strain recovered in low numbers from heart blood.

TABLE 5. *Ability of 31 strains of Y. enterocolitica to induce conjunctivitis in guinea pigs*

Culture no.[a]	Serotype O:	Dose (cells)	Response in animal no.[b]:		
			1	2	3
E235	3	8.7×10^8	−	−	
E546	3	1.2×10^9	±	±	
E547	3	9.0×10^8	+	−	
E549	3	1.4×10^9	±	±	±
E162	5	8.9×10^8	−	−	
E559	5	5.6×10^8	−	−	
E560	5	1.0×10^9	−	−	
E543	8	2.6×10^9	±	±	
E557	8	1.6×10^9	++	++	++
E558	8	1.6×10^9	++	++	
E565	8	3.8×10^8	−	−	
E572	8(R)	1.8×10^9	++	++	
E049	16	1.6×10^9	−	−	
E202	16	6.9×10^8	−	−	
E561	4,32	1.1×10^9	−	−	
E562	4,32	1.2×10^9	−	−	
E619	4,33	1.2×10^9	−	−	
E083	5,27	5.4×10^8	±	±	
E544	5,27	1.8×10^9	±	±	
E555	5,27	9.6×10^8	±	+	+
E556	5,27	1.3×10^9	±	±	
E545	6,30	1.4×10^9	±	−	−
E612	6,30	5.0×10^8	−	−	
E616	6,31	7.1×10^8	−	−	
E620	7,8	7.5×10^8	−	−	
E119	13,7	5.1×10^8	−	−	
E563	13,7	8.4×10^8	−	−	
E564	13,7	6.9×10^8	−	−	
E277	NT[c]	5.1×10^8	−	−	
E626	NT	5.3×10^8	−	−	
E629	NT	5.7×10^8	−	−	

[a] See Table 1 for source of cultures.
[b] See text for explanation of response recorded.
[c] NT, Nontypable.

TABLE 4. *Distribution of Y. enterocolitica in gerbils after fatal infection by i.p. or oral route of administration*

Culture no. (serotype)	Route	Animal no.	*Y. enterocolitica* isolated from[a]:					
			Heart blood	Lung	Liver	Spleen	Feces	Intestinal fluid
E557 (O:8)	i.p.	1	++++	+++	++++	++++	ND	ND
		2	+++	+++	+++	+++	ND	++
	Oral	1	++++	++++	++	+++	−	−
		2	+++	++	+	+	+	ND
E572 (O:8R)	i.p.	1	++++	+?	++++	++++	ND	ND
		2	+++	+?	++++	++++	ND	++++
	Oral	1	+++	ND	+	++++	−	ND
		2	++++	++++	+	++++	−	ND

[a] ND, Not done; +?, hemorrhaging in thoracic cavity made recovery from lungs questionable; +, ++, +++, ++++, relative growth on MacConkey agar from a streaked homogenate.

four strains of serotype O:5,27 generated either weak (±) or mild (+) reactions. Three of four strains of serotype O:3 gave reactions, but in one case in only one of the two animals exposed and with only weak (±) reactions for the remaining two cultures. Strains of serotype O:8 induced the strongest and most reproducible infections. However, two of the five strains of serotype O:8 were completely negative for guinea pig conjunctivitis, suggesting that virulence is not uniform among strains of this serotype.

Virulence and VW antigen. After completing the gerbil and guinea pig studies (Tables 1 and 5), we looked for a possible correlation between virulence and the presence of VW antigen as determined by the inability to grow on calcium-deficient MOX agar at 35°C. When a high correlation was found retrospectively, a controlled experiment was conducted with simultaneous administration of the organism to guinea pigs and gerbils. The results for 12 strains of *Y. enterocolitica* (Table 6) show a positive prediction of virulence by cultural determination of the VW antigen for four of the five serotypes examined. The exception was serotype O:3, which possessed the VW antigen according to differential growth on the TSA-MOX agar system but was not consistently virulent by either animal test.

Two cultures which were previously tested in guinea pigs and gerbils gave contradictory results on retesting. The first culture (E543, sero-

TABLE 6. *Relationship of Y. enterocolitica virulence in gerbils and guinea pigs to a calcium requirement for growth*

Culture no.[a]	Serotype O:	Growth[b] on.:		Gerbil lethality			Guinea pig conjunctivitis		
		TSA	MOX	Animal no.	Dose (cells)	Result	Animal no.	Dose (cells)	Result[c]
E543	8	+	+	1	9.1×10^7		1	1.8×10^9	−
				2	9.1×10^7		2	1.8×10^9	−
E547	3	+	−	1	8.0×10^6		1	1.6×10^8	±
				2	8.0×10^6		2	1.6×10^8	+
E549	3	+	−	1	4.5×10^7		1	9.0×10^8	−
				2	4.5×10^7		2	9.0×10^8	−
E555	5,27	+	+	1	7.4×10^7		1	1.5×10^9	−
				2	7.4×10^7		2	1.5×10^9	−
E557	8	+	−	1	3.2×10^7	Death	1	6.4×10^8	++
				2	3.2×10^7	Death	2	6.4×10^8	++
E558	8	+	−	1	4.0×10^7	Death	1	8.1×10^8	++
				2	4.0×10^7	Death	2	8.1×10^8	++
E560	5	+	+	1	6.3×10^7		1	1.3×10^9	−
				2	6.3×10^7		2	1.3×10^9	−
E564	13,7	+	+	1	4.8×10^7		1	9.6×10^8	ND[d]
				2	4.8×10^7		2	9.6×10^8	−
E565	8	+	+	1	5.8×10^7		1	1.2×10^9	−
				2	5.8×10^7		2	1.2×10^9	−
E572	8(R)	+	−	1	1.0×10^7	Death	1	2.0×10^8	++
				2	1.0×10^7	Death	2	2.0×10^8	++
E619	4,33	+	+	1	4.4×10^7		1	$8.7 \times$ [illegible]	−
				2	4.4×10^7		2	$8.7 \times$ [illegible]	−
E644	8	+	−	1	1.2×10^7	Death	1	4.2×10^8	++
				2	1.2×10^7	Death	2	4.2×10^8	++

[a] See Table 1 for source of cultures.
[b] At 35°C.
[c] See text for explanation of response recorded.
[d] ND, No data.

type O:8) gave positive reactions for guinea pig conjunctivitis and gerbil lethality the first time but negative results the second; the second culture (E619, serotype O:4,33) gave a negative test for guinea pig conjunctivitis and positive for gerbil lethality the first time but negative reactions for both tests the second time. The negative results upon retesting were consistent with the absence of VW antigen according to inability to grow on MOX agar. The inconsistencies between experiments may result from different methods used for preparing the cells, loss of virulence through subculturing, or because a population of cells contains both virulent and avirulent forms. The first experiments were done with mixed broth cultures and the second with growth from an agar medium inoculated with cells from a single colony.

DISCUSSION

Virulence of *Y. enterocolitica*, as measured by both the Serény test, in which it produced only conjunctivitis and not the keratoconjunctivitis observed with shigellae (20), and the gerbil lethality test, was restricted to a few serotypes. Some but not all strains of serotype O:8 demonstrated the highest virulence in both animal systems. Our results agree with those reported by Feeley et al. (7), who found that positive Serény tests were obtained with strains of serotype O:8 only. The absence of virulence in certain strains of serotype O:8 may be due to the fact that this property is plasmid mediated (23) and therefore can be lost with subculture. Gerbils were particularly vulnerable to virulent strains of serotype O:8 by either i.p. or oral routes of administration with very low doses. This virulence was impressed upon us by the troublesome transmission encountered during these studies between animals infected with serotype O:8 and animals infected with other types and physically isolated in separate cages but held on the same shelf unit.

The most common human serotypes isolated in Canada, i.e., O:3, O:5,27, and O:6,30, were essentially avirulent in both animal tests. Strains of serotype O:3 were uniformly avirulent by the gerbil test and produced only mild or inconsistent reactions in the guinea pig conjunctivitis test. Strains of serotype O:5,27 were more consistent in guinea pigs and mostly avirulent in gerbils, whereas strains of O:6,30 were essentially avirulent by both test systems. These results suggest that the guinea pig conjunctivitis test is a more sensitive system than the gerbil test for detecting virulence; however, the results are not always conclusive. For the other strains of *Y. enterocolitica* examined, the two animal systems were basically in agreement.

The absence of or low virulence in strains of serotype O:3, which predominate in human infections in Canada and many other countries, suggests that another mechanism of pathogenesis may be associated with these organisms. Strains of serotype O:3 commonly produce a heat-stable enterotoxin (14, 18a). Because this toxin cannot be produced in vitro at body temperature (13), its role in pathogenesis has been questioned and is still unresolved. If the role of this toxin is eventually established, then it may be that *Y. enterocolitica*, like *Escherichia coli* (15, 18), can be divided into two groups with respect to mechanism of pathogenesis, those that are enteroinvasive and those that are enterotoxigenic.

The use of MOX agar for determining a calcium requirement for growth, which has been associated with the presence of VW antigen, is a uniquely simple technique for predicting virulence, particularly for strains of serotype O:8. The inability of strains of serotype O:3 to grow on MOX agar at 35°C suggests that these organisms also possess the VW antigen, and yet they were mostly avirulent by the gerbil and guinea pig conjunctivitis test systems. Whether this inability to grow on MOX agar at 35°C is truly associated with the presence of the VW antigen or is merely a reflection of intolerance to magnesium or the higher temperature or both remains to be determined.

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ADDENDUM

Subsequent to submission of this manuscript, Gemski et al. (Infect. Immun. **27**:682–685, 1980) reported that virulence in three strains of *Y. enterocolitica* serotype O:8, demonstrated by the Serény test, was associated with a plasmid which also coded for calcium dependency.

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