INFECTION AND IMMUNITY, Nov. 1981, p. 550-560
0019-9567/81/110550-11$02.00/0

Vol. 34, No. 2

Evaluation of Iron Dextran and Mucin for Enhancement of the Virulence of *Yersinia enterocolitica* Serotype O:3 in Mice

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Received 1 April 1981/Accepted 30 June 1981

The pathogenic role of *Yersinia enterocolitica* serotypes O:3, O:8, and O:9 in human infections is well documented. Whereas the virulence of the O:8 strains can be readily demonstrated in mice by 50% lethal dose determinations, the O:3 and O:9 strains have no lethal effect on mice by any route of inoculation. A mouse virulence test for the O:3 and O:9 strains is described. *Y. enterocolitica* strains were first tested for the presence of virulence-associated plasmid characteristics by auto-agglutination and gel electrophoresis procedures before mouse virulence determinations. The 50% lethal dose of the O:3 strains injected intraperitoneally with 2.5% mucin was about 10^7 colony-forming units. However, histological examinations showed that mucin allowed the growth of *Y. enterocolitica* on the surface of the livers and spleens of the mice without internal lesions. The 50% lethal dose of the same O:3 strains injected intraperitoneally with 1 ml of 10% iron dextran in saline was about 10^5 to 10^6 colony-forming units, and the nonlethal infective dose with typical lesion development was 20 to 200 colony-forming units. The infected mice developed symptoms and extensive liver and spleen lesions which differed from those in mice infected intraperitoneally with the virulent O:8 strains. These results showed that the virulence of the O:3 *Y. enterocolitica* strains can be measured by intraperitoneal injection with iron dextran. This procedure was used to test the virulence of food isolates, plasmidless strains, and the effect of growth temperatures.

Yersinia enterocolitica causes illnesses in humans (6, 44) and in animals (17, 43). It is also commonly recovered from certain foods, such as raw meats, mussels, oysters, salad vegetables, and water (22, 36). Its presence in foods is of serious public health concern because it is one of the few human pathogens that can grow at the proper food refrigeration temperatures of 0 to 5°C. For this reason, reliable methods are needed to assess the virulence potential of *Y. enterocolitica* food isolates. The species is a heterogeneous collection of strains that are not closely related (7). It is generally agreed that most of the food isolates belong to the atypical, rhamnose-fermenting strains or salicin- and esculin-fermenting Nilehn (28) biotype 1 strains. The clinical symptoms and probably the virulence of the atypical strains and the Nilehn biotype 1 strains are much milder and completely different than those of the typical clinical strains. The potential virulence of the atypical strains should be investigated separately to avoid confusion.

Perhaps less well known is the fact that considerable differences in bacterial physiology and laboratory animal virulence also exist among the typical serotype O:3, O:8, and O:9 human clinical isolates which are closely related by deoxyribonucleic acid homology (7, 8). The majority of the clinical strains isolated worldwide grow in strict or inhibitory selective media such as the $MgCl_2$ (41) and selenite (40) enrichment broths; are resistant to pesticin (19); are ST positive (27, 29, 31); are Sereny negative; and have no effect on mice by intraperitoneal injection (1). The typical strains of this group are the serotype O:3 Nilehn biotype 4 MCH76 and MY778 strains that were investigated in this study. Another group of clinical strains is sensitive and does not grow in strictly selective media as mentioned above, is susceptible to pesticin (12, 19), is virulent to guinea pigs by the Sereny test (14), and is lethal to mice (10, 11, 33). The typical strains in this group are the serotype O:8 Nilehn biotype 2 strains, such as the strain CDC A2635 isolated from contaminated chocolate milk (14) and the WA strain (10, 11). The differences in laboratory animal virulence among the serotype O:3, O:8,

and O:9 strains are not reflected in humans since all three serotypes have caused similar clinical symptoms of nonlethal infections in humans (6, 44). Recently the presence of a 41- to 42-megadalton (Mdal) virulence-associated plasmid was discovered in the serotype O:8 mouse-lethal strains (16, 46). The properties of autoagglutination (21), calcium dependency (12, 16), and the production of V and W virulence factors at 35°C (12) correlate with the virulence-associated plasmid. In this study, these tests and the presence of a virulence-associated plasmid were applied to the serotype O:3 and O:9 *Y. enterocolitica* strains.

Different procedures have been used to enhance the virulence of the serotype O:3 biotype 4 strains. Among these are stressed mice (21), germfree mice (4), nude mice (1), irradiated mice (45), cyclophosphamide-treated mice (2), and ferrous ammonium citrate-treated mice (34, 35). Also, Une (39) and Pai et al. (30) developed a rabbit diarrhea test for these biotypes. All of these methods are qualitative tests, although Robins-Browne et al. (35) presented evidence that suggested a dose-dependent mortality from serotype O:3 *Y. enterocolitica* injection after administration of ferric ammonium citrate. To study the relative differences in virulence due to genetic, environmental, or biochemical influences, a quantitative animal virulence test is needed.

Mucin (9, 13) and iron dextran (3, 9, 20, 38) have been used to enhance the virulence of bacteria other than yersiniae. These compounds have relatively low toxicity for mice. In this report, mucin and iron dextran were evaluated for the ability to enhance the virulence of fresh clinical and food isolates of *Y. enterocolitica* strains for mice as a means of developing an animal test system that is selective and quantitative.

MATERIALS AND METHODS

Bacterial strains. Freshly isolated clinical *Y. enterocolitica* serotype O:3 biotype 4 isolates denoted with the "MCH" prefix were obtained from C. H. Pai, Montreal Children's Hospital, Montreal, Canada. Strains denoted with a "MY" prefix were supplied by S. Winblad, University of Lund, Malmo, Sweden. The serotype O:9 E265 and W770 strains were obtained from G. Wauters, Clinques Universitaires St. Luc, Brussels, Belgium. The mouse-lethal serotype O:8, Nilehn biotype 2 CDC A2635 strain, which was isolated from a carton of chocolate milk during an outbreak of yersiniosis, was obtained from J. C. Feeley (14), Center for Disease Control, Atlanta. Plasmidless control strains were obtained as follows: MCH76$^-$ is a spontaneously derived plasmidless MCH76 strain isolated in our laboratory by an auto-agglutination procedure; MCH628$^-$ was derived in the same manner and was obtained from W. J. Laird, Bureau of Biologics, Bethesda, Md.; TAMU75$^-$ (CDC A2635$^-$), obtained from D. L. Zink, Texas A & M University, College Station, Tex., was induced by incubation at 35°C (46). A plasmidless *Escherichia coli* K-12 strain 711 was obtained from R. P. Silver, Bureau of Biologics, to assess the effect of iron dextran on a known avirulent *E. coli* strain. *Y. enterocolitica* strain 4-4 was isolated from a pig's throat by N. J. Stern, U.S. Department of Agriculture-S.E.A., Beltsville, Md. All food isolates were recovered by the F.S.Q.S. laboratories. For convenience, all *Y. enterocolitica* strains lacking the virulence-associated characteristics of auto-agglutination and calcium dependency are designated with a "−" superscript. Cultures were stored in the form of a thick bacterial suspension in a solution of 30% glycerol and 1% peptone (24) at −20 or −70°C to preserve the plasmid.

Plasmid screening and detection. All cultures were routinely screened for the presence of the virulence-associated plasmid by testing 20 isolated colonies by an auto-agglutination screening test (21). Calcium dependency or inability to grow on magnesium oxalate agar was tested by published procedures (16, 21). *Y. enterocolitica* plasmids were extracted by the method of Hansen and Olsen (18) by using extended exposure to lysozyme and ethylenediaminetetraacetic acid (30 min at 37°C), but omitting the alkaline denaturation step. Plasmids were separated in a horizontal 0.7% agarose gel (6 by 200 by 250 mm; medium EEQ; Sigma Chemical Co., St. Louis, Mo.) with tris(hydroxymethyl)aminomethane-borate-ethylenediaminetetraacetic acid buffer (46) at 10 to 15 V/cm for 4 to 6 h. The sizes of the plasmids were determined by comparison with the log-log plots of the following standard reference plasmids in *E. coli* strains: R6-5 (TAMU146), Sa (TAMU149), ColE1 (TAMU154) (46), and pVA517A-H (*E. coli* V517) (25).

Mice. Outbred Swiss male mice, 17 to 20 g, were obtained from Microbiological Associates (MAI), Bethesda, Md.; Charles River Breeding Lab (CRL), Wilmington, Mass.; and the National Institutes of Health (NIH), Bethesda, Md. Different mouse sources were used for comparative purposes, or from necessity of supply, but only a single source of mice was used in each experiment. Mice were fed and watered ad libitum, and food was dropped to the cage floor for obviously sick mice. Guinea pigs were obtained from Charles River Breeding Laboratories.

Intraperitoneal infection procedure. Groups of six mice per dilution were used to assess the virulence of *Y. enterocolitica* strains by intraperitoneal injection. Strains used for virulence testing were first selected for motility and then grown on thick (30 ml/100-mm plate) Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) as described previously (24). Unless specified otherwise, the bacteria were grown at 5°C for 5 days and then at 22°C for 1 day. Cultures were harvested by suspending the bacteria in saline, and the optical density was adjusted to 2 in 13-mm round cuvettes at 600 nm. The original bacterial saline suspensions were diluted in saline and counted for total cell number in a Petroff-Hauser counting

chamber, and the colony-forming units (CFU) were determined by pour plate in Trypticase soy agar. Decimal dilutions of the optical density 2 suspensions (about 4×10^9 CFU) were made with saline and mixed with equal volumes of sterilized saline, 5% hog gastric mucin in saline (pH 7.4) (Sigma), or 20% iron dextran (Imferon; Merril-Richardson Inc., Cincinnati, Ohio) in saline for intraperitoneal injections. For 50% lethal dose (LD_{50}) determination, groups of six mice were each injected intraperitoneally with 1 ml of each of the dilutions of the bacterial suspension prepared as indicated above. Heat-killed suspensions which had been boiled for 5 min were also injected to test for possible endotoxin death.

Sereny test for invasiveness. A drop of *Y. enterocolitica* suspension containing about 2×10^{10} CFU/ml in saline or in 10% iron dextran in saline was placed onto the conjunctival membrane of one eye of a male guinea pig weighing 250 g (37). The other eye of the animal was administered a drop of either saline or 10% iron dextran in saline (whichever solution was used to dilute the test bacteria) as a control. Strains MCH76 and MY778 were tested by this method, and a known Sereny-positive strain, CDC A2635 (14, 46), was included as a control.

Mortality and morbidity observations and calculations. Infected mice were observed for symptoms of lethargy, ruffled fur, closed eyes, diarrhea, fecal excretion of *Y. enterocolitica*, and death for 21 days. The shape, color, and size of the stools were observed by placing the infected mice in a clean cage lined with a clean piece of white paper. Totally unformed stools having a light color and semi-solid consistency were considered diarrhea.

In addition to the mice used for LD_{50} determinations, other infected mice were sacrificed at intervals or when ill to examine pathological findings. Dead mice were autopsied as soon as possible. After gross pathology was noted, sections of the spleen, liver, and lymph were taken for histological examination. The tissues were fixed in 10% buffered Formalin, embedded in paraffin, sectioned to 6-μm thickness, and then stained with a hematoxylin and eosin stain. Other sections from the same tissue were stained with modified Lillie-Twort strain (Claude West, personal communication), designed to stain the gram-negative bacteria red in tissue sections.

The LD_{50} and the 95% confidence limits were calculated from cumulative mortality data by the "moving averages" method (26) and confirmed by a computerized version of Finney's (15) probit transformation. The two methods were generally in agreement.

To recover *Y. enterocolitica*, infected organs were placed in sterile whirlpak bags (Nasco, Ft. Atkinson, Wis.) containing 5 ml of sterile distilled water and homogenized in a Colworth Stomacher 80 (Dynatech Laboratory, Alexandria, Va.). Stool samples were also taken at intervals and dispersed in a tube with 5 ml of sterile distilled water. The stools and tissue suspensions were then streaked for isolation of the organism on deoxyribonuclease agar with sorbitol and Tween 80 (23). Representative colonies were identified by reactions on triple sugar iron slants, oxidase test, and the API-20E System (Analytab Products, Plainview, N.Y.).

RESULTS

Auto-agglutination, calcium dependency, and plasmids in serotype O:3 and O:9 *Y. enterocolitica* strains. The virulence-associated properties mentioned previously and plasmids found in the serotype O:8 strains (12, 16, 21, 46) were assessed in all the *Y. enterocolitica* strains in our collection. The old laboratory stock cultures, such as the IP107, IP134, IP383, and MY79b reference serotype strains, were invariably negative by auto-agglutination, calcium dependency, and gel electrophoresis tests. About 20 fresh clinical serotype O:3 and O:9 strains obtained from Canada, Belgium, and Sweden were nearly all positive by auto-agglutination and calcium dependency tests (Table 1). Except for the E265 strain, all of the other auto-agglutination positive serotypes O:3 and O:9 strains were shown to possess only a single plasmid by gel electrophoresis (Table 1). The presence of this plasmid also correlated to calcium dependency tests (Table 1). Thus, it appeared that the serotypes O:3 strains possessed a plasmid with similar properties but slightly larger size than the serotype O:8 pDZ19 plasmid (46), the VWA plasmid (16), and the plasmid detected in serotype O:9 strains (46 versus 42 Mdal). The two sizes of plasmids consistently separated by electrophoresis when both were applied to the same well in the gel bed. Two food isolates of porcine origin also possessed a 46-Mdal plasmid (Table 2). The possible virulence properties associated with the 42- and 46-Mdal plasmids were investigated.

Mouse infection by intraperitoneal injection with mucin and saline. As previously mentioned, it is well established that the serotype O:3 and O:9 strains consistently failed to cause infection in mice (1) or in guinea pigs (27) by a variety of inoculation techniques. However, these tests were performed before a virulence-associated plasmid was known to exist in *Y. enterocolitica* (16, 46), and thus it is not certain whether the strains tested contained a virulence-associated plasmid. The test with saline intraperitoneal injections was conducted with serotype O:3 strains, which were positive for auto-agglutination and calcium-dependent properties and contained a 46-Mdal plasmid. An inoculum of 10^8 CFU of serotype O:3 strains failed to elicit any ill effect in mice. Mucin and iron dextran were then tested to see whether either of these compounds could potentiate the virulence of these plasmid-containing serotype O:3 and O:9 strains.

Intraperitoneal injection with 2.5% hog gastric mucin in saline did enhance the virulence of O:3 MCH76 (Table 1) and MCH628 strains. How-

TABLE 1. *Some properties of clinical Y. enterocolitica strains and their relation to mortality in mice from intraperitoneal injection with mucin and iron dextran*

Strain	Serotype	Source	ST[a]	Plasmid size (Mdal)	Auto-agglutination	Calcium dependency	LD_{50} (CFU) when injected with:	
							2.5% Mucin	10% Iron dextran
MCH76	O:3	Canada	+	46	+	+	1.3×10^7	$2.3 \times 10^{5b,c}$
MCH76$^-$	O:3	Canada	+	None	−	−	2×10^8	$>2.3 \times 10^{7d}$
MCH628$^-$	O:3	Canada	+	None	−	−	2×10^8	1.2×10^{8e}
MY778	O:3	Sweden	+	46	+	+	NT[f]	2.3×10^{5g}
E265	O:9	Belgium	+	36, 42	+	+	NT	2.3×10^{6h}
W770	O:9	Belgium	NT	42	+	+	NT	1.3×10^{7h}
IP383$^-$	O:9	Belgium	NT	None	−	−	3×10^7	7.1×10^{6e}
MY79b	O:9	Sweden	NT	None	−	−	NT	$>2.6 \times 10^{7h}$
E. coli	NA[i]	NA	None	None	NA	NA	NT	$>10^7$

[a] Tested by W. J. Laird.
[b] Average of 3 tests.
[c] MAI, NIH, and CRL mice used.
[d] MAI and NIH mice used.
[e] MAI mice used.
[f] NT, Not tested.
[g] NIH mice used.
[h] CRL mice used.
[i] NA, Not applicable.

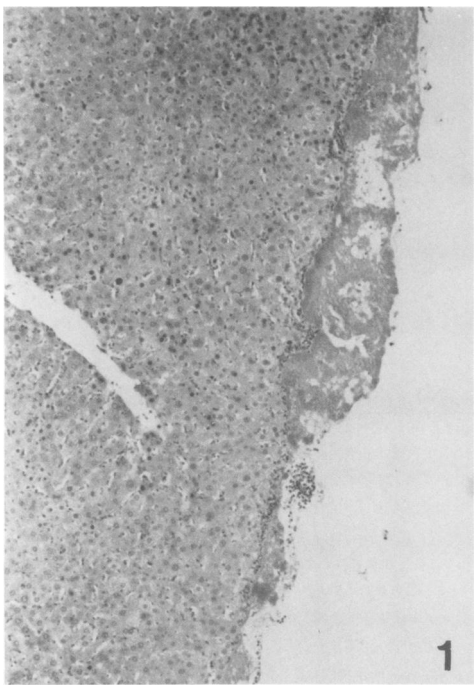
TABLE 2. *Virulence potential of Y. enterocolitica food and animal isolates*

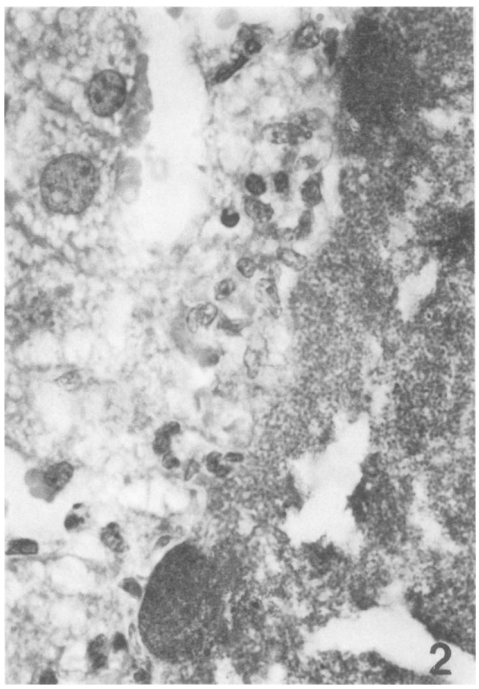
Strain	Source	Nilehn biotype	Auto-agglutination	Plasmid size (Mdal)	LD_{50} (CFU) when injected with:	
					Saline	Iron dextran
A78-5	Beef	1	−	32	No[a]	No
A78-34	Beef	1	−	32	No	No
A78-42	Beef	Rham[b]	−	32	No	No
A78-92	Beef	Rham	−	15	No	No
A78-465	Beef	Rham	−	3,4,11,36	No	No
78-513	Pork	3	+	46	No	2×10^5
4-4	Pig's throat	3	+	46	No	1.3×10^5

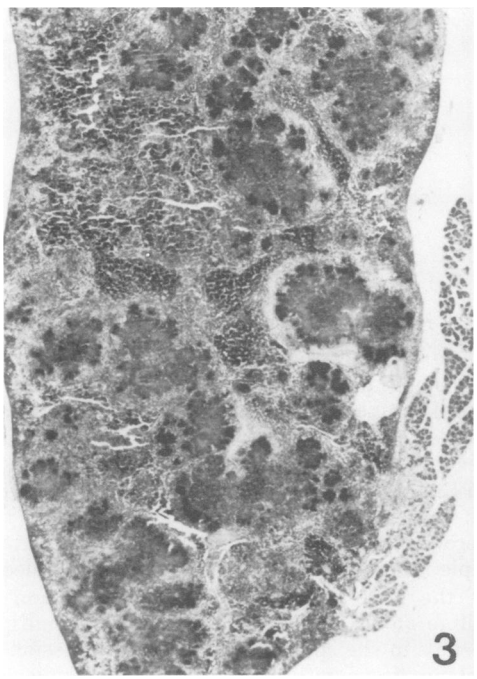
[a] No, No death or illness observed; $LD_{50} > 2 \times 10^8$.
[b] Rham, Ferments rhamnose.

ever, the virulence of the plasmidless strain (IP383$^-$) was also enhanced by mucin to nearly the same degree as the plasmid-containing strains (Table 1). Histopathological examination revealed that mucin allowed the profuse growth of *Y. enterocolitica* on the surfaces of livers (Fig. 1) and spleens without any internal lesion development. Figure 2 is a higher magnification of the liver section of a mouse injected with the MCH76 strain and mucin. It shows the growth of the *Y. enterocolitica* colonies on the surface of the liver. Also, Fig. 2 shows an early inflammatory response consisting primarily of neutrophils along the sites of bacterial infection. These figures clearly demonstrate that mucin promoted a general peritonitis by the O:3 *Y. enterocolitica* strains and did not promote any internal lesion development typical of serotype O:8 mouse infection (11).

To compare to serotypes O:3 and O:9 infections with mucin and iron dextran, the plasmid-containing serotype O:8 CDC A2635 strain and a plasmidless derivitive (TAMU75) were injected intraperitoneally with saline. Differences in LD_{50} (Table 3) and symptoms were observed. In contrast to mucin-enhanced O:3 infection, the serotype O:8 CDC A2635 strain produced multiple internal abcesses in the livers and spleens of the mice after intraperitoneal injection in saline alone (Fig. 3). Figure 3 shows multiple lesions in the spleen in addition to adhesion of splenic tissue with pancreatic tissue. Typically, mice infected with this strain become ruffled and lethargic 5 to 6 days after injection. The LD_{50} of the CDC A2635 strain was only 70 CFU when injected with saline (Table 3). The plasmidless derivative of CDC A2635 strain (TAMU75) (46) did not cause any morbidity or







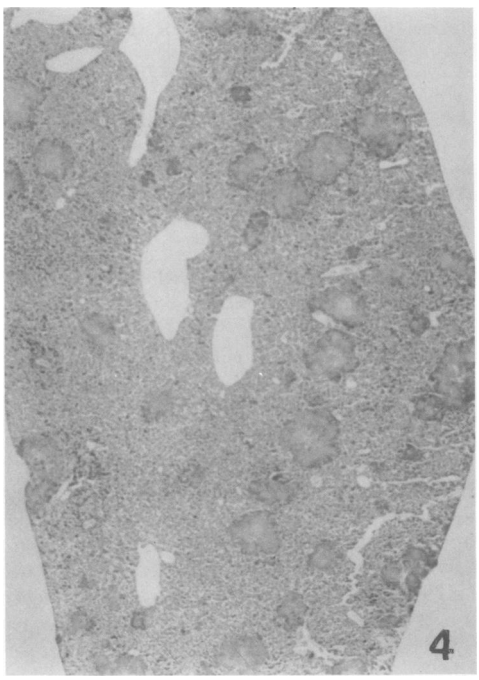


FIG. 1. *Colonies of Y. enterocolitica growing on the surface of a liver section from a mouse after intraperitoneal inoculation with strain MCH76 in hog gastric mucin.*

FIG. 2. *Early inflammatory response along growth of Y. enterocolitica on the surface of a liver section from a mouse inoculated intraperitoneally with strain MCH76 in hog gastric mucin.*

FIG. 3. *Multiple spleen lesions and adhesion of splenic tissue to pancreatic tissue in a section from a mouse after intraperitoneal inoculation with the mouse-lethal serotype O:8 strain CDC A2635 in saline.*

FIG. 4. *Multiple liver lesions in a mouse inoculated intraperitoneally with Y. enterocolitica strain MCH76 in iron dextran.*

TABLE 3. *LD_{50} of Y. enterocolitica strains grown at 5, 22, or 35°C*

Strain	Growth temp (°C)	Days	LD_{50} (CFU)	95% Confidence limits (CFU)
CDC A2635 (in saline)	5	14	170	48–530
	22	1	70	22–220
	35	1	100	NC[a]
MCH76 (in iron dextran)	5	14	1,900	NC
	22	1	6,600	1,000–43,000
	35	1	8,000	1,400–60,000

[a] NC, Not calculable.

mortality in mice when injected in saline intraperitoneally.

Mouse infection by intraperitoneal injection with iron dextran. Iron dextran enhanced the virulence of the *Y. enterocolitica* strains to a much greater degree than did mucin (Table 1). It also enhanced the lethality of the plasmid-containing serotype O:3 MCH76 and the MY778 strains to a much greater degree ($P < 0.05$) than the plasmidless serotype O:3 MCH76$^-$ and MCH628$^-$ isolates (Table 1). Similar LD_{50} (1×10^5, 2×10^5, and 5×10^5 CFU) for the MCH76 strain were obtained with CRL, NIH, and MAI mice. However, one lot of MAI mice was very sensitive to MCH76 infection (LD_{50}, 10^4 CFU) for reasons not known (Table 3). The 95% confidence limits of LD_{50} data calculated from experiments using groups of six mice ranged from 1 to 2.5 log CFU. The wide range of confidence limits apparently resulted from tailing of the mortality data at the low doses (CFU).

Intraperitoneal injection of the MCH76 and MY778 strains produced distinct nonlethal infections at very low doses. The nonlethal infective dose was only about 20 to 200 CFU in the intraperitoneal iron dextran system. Mice infected with these low doses still developed symptoms of ruffled fur, diarrhea, and internal liver and spleen lesions comparable to those produced by lethal infections at higher doses. Internal liver lesions were observed in mice infected with 200 CFU of the MCH76 strain from 1 to 3 weeks post-inoculation. The external symptoms of the sick mice generally disappeared within 2 to 3 weeks, and these mice eventually recovered from the infection. Autopsy of mice killed from MCH76 infection after intraperitoneal iron dextran inoculation revealed multifocal liver and spleen lesions accompanied by enlarged spleens and tissue adhesion. Histopathological examinations of the infected mice showed the development of liver necrosis (Fig. 4) similar to that seen in mice infected by the mouse-lethal CDC A2635 strain (Fig. 3). Figure 5 is a higher magnification of a liver section showing an early inflammatory response to infection enhanced by iron dextran, and internal *Y. enterocolitica* growth (arrow). However, no systemic infection like that caused by the CDC A2635 strain in mice was observed.

Iron dextran also enhanced the virulence of the serotype O:9 strains. However, the E265 and W770 strains with the 42-Mdal plasmid were less virulent to mice when injected intraperitoneally with iron dextran than were the serotype O:3 strains (Table 1). Infection with the plasmid-containing E265 and W770 strains produced symptoms of ruffled fur, diarrhea, internal liver lesions, and death with a time course identical to that produced by the plasmid-containing serotype O:3 strains. Infection with the plasmidless IP383$^-$ strain produced no internal lesions, and most of the deaths occured on day 1 after inoculation. No significant difference in mortality can be observed among the serotype O:9 E265, W770, IP383$^-$, and MY79b strains. Iron dextran injection did not promote mouse infection by the *E. coli* K-12 strain 711 (Table 1).

Toxicity. Heat-killed suspensions of 10^9 CFU of *Y. enterocolitica* strains in saline, mucin, or iron dextran were found to kill mice when injected intraperitoneally. This toxic effect was absent from heat-killed suspensions of 10^8 CFU or less. Therefore, only data corresponding to mouse infection from 10^8 CFU or less were considered in morbidity and mortality determinations. Comparative total and CFU counts indicated that more than 60% of the bacterial cells in suspensions used for injections were viable; therefore, the toxic effect of dead bacteria in these suspensions at 10^8 CFU or less was not a significant factor in the mortality results presented in Tables 1, 2, and 3.

Sereny test. Two attempts were made to induce conjunctivitis in guinea pigs' eyes with the MCH76 and the MY778 strains suspended in saline as well as 10% iron dextran in saline. The results were negative, but the positive control test with the CDC A2635 strain in saline caused typical conjunctivitis in the guinea pig eye within 4 days (14).

Onset of symptoms and mortality in infected mice. Mice infected with various virulence plasmid-containing or plasmidless clinical strains developed distinctive symptoms and a pattern of mortality characteristic of one of three types. Injection of 10^4 CFU of plasmid-containing serotype O:3 and O:9 strains (e.g., MCH76 and E265, respectively) in iron dextran caused morbidity and mortality in mice beginning on day 1 after injection and lasting up to 10 days postinjection. Infected mice typically experienced a progressive symptomatology consisting of increasing degrees of ruffling, lethargy, and labored breathing. Deaths occurred primarily

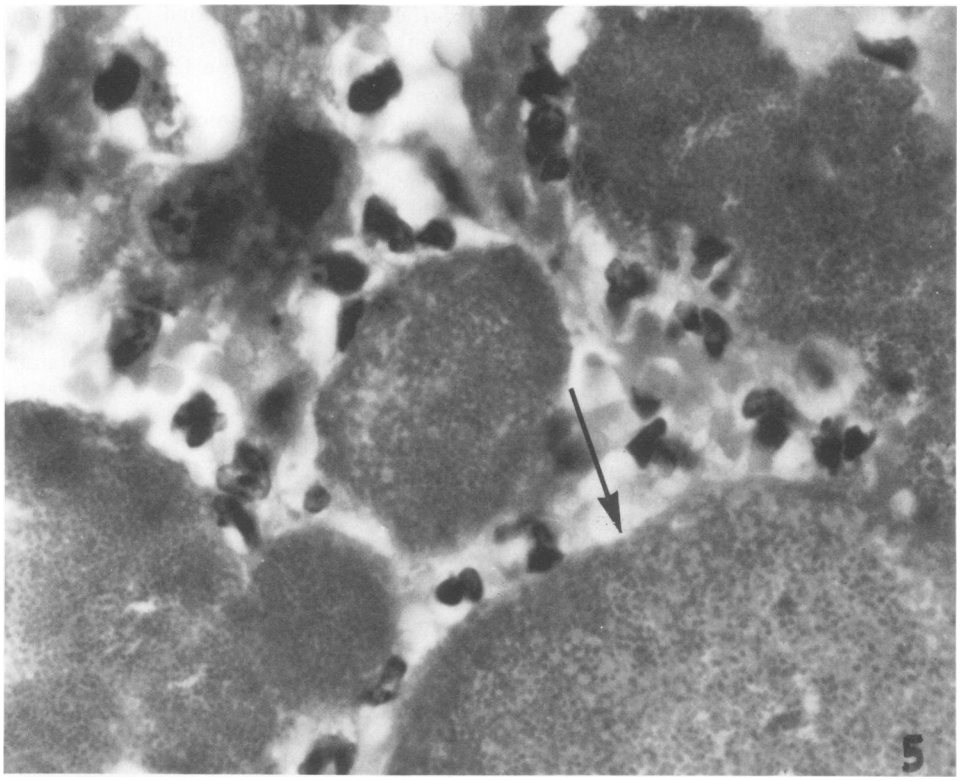


FIG. 5. *Colony of Y. enterocolitica (arrow) and early inflammatory response in a liver section from a mouse after intraperitoneal inoculation of strain MCH76 in iron dextran.*

beyond day 1 postinjection and continued for several days. Fecal shedding of *Y. enterocolitica* persisted for up to 21 days after injection. By contrast, injection of 10^4 CFU of plasmidless O:3 and O:9 strains in iron dextran caused no infection in mice. At a dose of 10^7 CFU, one of these strains (IP383$^-$) caused illness and some mortality. However, in all cases, more than 80% of deaths caused by IP383$^-$ occurred within the first 24 h after injection. Fecal shedding of IP383$^-$ did not persist beyond the first few days after injection.

Infection in mice caused by the injection of 10^2 or 10^4 CFU of the serotype O:8 CDC A2635 strain had a delayed onset. Symptoms and mortality did not occur until day 4 postinjection and beyond. Shedding of *Y. enterocolitica* in feces persisted for up to 30 days.

Effects of varied growth temperatures on the virulence potential of *Y. enterocolitica*. With the development of a practical mouse virulence test procedure for the serotype O:3 strains, it was possible to measure the virulence potential of the serotype O:3 and O:8 strains grown at 5, 22, and 35°C (Table 3). No significant difference in the LD_{50} data was observed for either serotype O:3 or O:8 inocula grown at the different temperatures (Table 3).

Virulence of food isolates for mice. The various virulence test procedures were also applied to various food isolates of *Y. enterocolitica* not connected with human illnesses. The food isolates A78-42, A78-92, and A78-465 were invasive to HeLa cells (22; unpublished results), but did not possess the 41- to 42-Mdal plasmid, nor were they virulent to mice (Table 2). On the other hand, the two pork and pig isolates (78-513 and 4-4) did possess the 46-Mdal plasmid and were virulent to mice by oral administration (21) and by intraperitoneal injection in 10% iron dextran (Table 2). By this method, the virulence characteristics of these two Nilehn biotype 3 strains are identical to the virulence test results of the MCH76 and MY778 strains listed in Table 1. Strains 78-513 (serotype O:3) and 4-4 (serotype O:1,2,3,) induced liver lesions and symptoms in the infected mice which were identical to those observed after injection of the serotype O:3 strains in iron dextran.

DISCUSSION

Research on the virulence of *Y. enterocolitica* is somewhat complex because only the serotype

O:8 and similar strains (12, 16, 21, 33) are lethal to mice, whereas the majority of the human clinical serotype O:3 strains have no effect on laboratory animals (1). Also, the existence of a virulence-associated plasmid (16, 46) was unknown at the time of previous reports on animal virulence, so it is not possible to determine whether the LD_{50} data presented (34, 45) were derived from plasmid-containing or plasmidless strains.

Recently, tbe properties of animal virulence (16, 46), auto-agglutination (21), calcium dependency (16), and most likely the production of temperature-dependent V and W virulence factors (12) were found to be associated with a 41- to 42-Mdal plasmid in the serotype O:8 clinical strains of *Y. enterocolitica*, which are highly lethal to mice. Auto-agglutination (21) and calcium dependency screening (12, 16) were applied to our collection of *Y. enterocolitica* cultures. It was found that almost all of our old stock cultures did not auto-agglutinate or show dependency for calcium at 35°C, although these old stock cultures were definitely invasive to HeLa cells (24). The screening tests showed that about 20 strains of fresh clinical isolates belonging to serotypes O:3 and O:9 as well as two isolates of porcine origin were positive by the auto-agglutination and calcium dependency tests (Tables 1 and 2). Identification of possible plasmids in these strains by gel electrophoresis revealed the existence of a single 46-Mdal plasmid in four of these strains (serotype O:3) and a 42-Mdal plasmid in two clinical strains (serotype O:9) (Tables 1 and 2). Just like the serotype O:8 strains reported (16, 21, 46), the spontaneous loss of the 46-Mdal plasmid in the strains listed in Table 1 resulted in the concomitant loss of auto-agglutination and calcium dependency properties. Possible animal infection and specifically lethality to mice by the serotype O:3 and O:9 *Y. enterocolitica* strains with the 46- and 42-Mdal plasmids were investigated in this study.

Previous research showed that animals must be stressed before they become susceptible to serotype O:3 infections (1, 2, 4, 21, 30, 35). Une (39), Pai et al. (31), and Laird and Cavanaugh (21) were able to induce experimental diarrhea in rabbits and mice with the serotype O:3 strains. Furthermore, Laird and Cavanaugh (21) demonstrated that the diarrheal virulence of the serotype O:3 IP4052 strain from France (2) was associated with the auto-agglutination characteristic. However, none of the tests described above is quantitative or makes it possible to measure the relative virulence of *Y. enterocolitica* strains.

In our experiments, the serotype O:3 and O:9 ST-positive strains with either the 46- or 42-Mdal plasmid (Table 1) were not virulent at all for mice when injected intraperitoneally in saline; serotype O:3 strains completely failed to cause keratoconjunctivitis in the eye of guinea pigs. Clearly, these O:3 strains were quite different in regard to mouse virulence than the serotype O:8 WA (11), Y7P, or the CDC A2635 (16) strains, and infection of outbred Swiss mice by these O:3 strains required enhancement of lethality. Ideally, an enhancement procedure should have low toxicity for the test animal. Mucin has been used to enhance the virulence of *Neisseria* (9) and *Vibrio* (13). Iron dextran has been used to enhance the virulence of *Escherichia* (3, 38), *Neisseria* (9, 20), *Klebsiella, Pasteurella,* and *Salmonella* (38). These compounds have relatively low toxicity for mice at the dosages employed.

Intraperitoneal injection of the plasmid-positive serotype O:3 strains with 2.5% hog gastric mucin did increase the virulence of these strains (Table 1) over intraperitoneal injection by saline. However, mucin enhanced the virulence of the plasmid-positive and -negative strains to nearly the same degree (Table 1). Histopathological examinations of the infected mice showed that mucin only promoted the growth of *Y. enterocolitica* on the surface of the liver and spleen (Fig. 1 and 2). No internal lesions were observed upon autopsy. For these reasons, mucin was not satisfactory for enhancing the virulence of serotype O:3 and O:9 strains.

Intraperitoneal injection of the serotype O:3 and O:9 strains of *Y. enterocolitica* with 10% dextran in saline resulted in tissue invasion, development of multifocal internal lesions as shown in Fig. 4 and 5, enlarged spleens, and death in the infected mice. The nonlethal infective dose with the same symptoms and welldefined internal liver lesion development was only about 200 CFU. This nonlethal infective dose was much lower than the infective dose established by other means of induced infection previously reported (1, 2, 31, 35, 39, 45, 46) for serotypes O:3 and O:9 strains and was about equivalent to the LD_{50} dose (by intraperitoneal route of inoculation in saline) of the mouse-lethal strain CDC A2635 (Table 3). However, the iron dextran-induced lesions were localized, characteristic of a nonsepticemic infection, and displayed an inflammatory response typical of an earlier stage in the infection process (Fig. 4 and 5). There was no progressive, systemic bacteremic infection resulting in extensive lesion development in the liver, spleen, pancreas, and other tissues (Fig. 3). Mice infected with low doses of the serotype O:3 and O:9 strains with iron dextran generally recovered after 21 days. Intraperitoneal injection of the plasmidless se-

rotypes O:3 and O:9 strains in iron dextran failed to induce any lesion development. The LD_{50} of the plasmidless O:3 strains MCH76$^-$ and MCH628$^-$ were significantly greater (10^7 CFU) than the 46-Mdal plasmid-containing strains MCH76 and MY778 (Table 1) and deaths occurred mostly within 1 day after injection.

Although the serotype O:9 strains E265 and W770 caused symptoms and definite lesion development with iron dextran infection, death was spread over many dilutions and the LD_{50} data were not significantly different ($P < 0.05$) than the plasmidless serotype O:9 IP383$^-$ and MY796 strains (Table 1).

It is possible that some plasmidless strains like the IP383$^-$ strain (Table 1) may contain an unidentified toxin or a weak virulence factor. It should be emphasized, however, that the plasmidless serotype O:9 IP383$^-$ and MY79b strains never induced liver lesion development when injected with iron dextran as did the E265 and W770 strains. The 95% confidence limits of the LD_{50} data for iron dextran serotype O:3 infections were less precise (1.5 to 2 log CFU) than the 95% confidence limits range of about 1 log CFU of the CDC A2635 strain (Table 3) or comparable data presented by Quan et al. (33). Nonetheless, the iron dextran method represents a major improvement for gauging the virulence of the serotype O:3 strains over what is currently available (1, 2, 4, 21, 34, 35, 45).

For corroborative purposes, the LD_{50} data should be considered in conjunction with the incidence of internal pathology and fecal shedding in mice infected with serotype O:3 strains. Data should be accumulated over at least a 7-day period since early mortality could be nonspecific.

The LD_{50} determinations presented in Tables 1, 2, and 3 were not affected by the presence of endotoxins from nonviable cells since the bacterial suspensions used for LD_{50} determinations were about 65% viable. Also, the cell concentrations injected for LD_{50} data were at least 1 to 2 logs lower than the minimum number of dead *Y. enterocolitica* which elicit death in mice.

The role of iron dextran in promoting infection in mice is not known for certain. The compound might have provided a source of iron for growth of the serotype O:3 and O:9 strains, or it might have neutralized the macrophages of the reticuloendothelial system of the mice as suggested by Suveges and Glavits (38). The serotype O:3 strains were noninvasive by the guinea pig conjunctiva invasiveness test when inoculated in suspensions of 10% iron dextran. This may suggest that the iron dextran was not just supplying iron nutrient to the O:3 strains, but also compromising some facet of host anti-bacterial reticuloendothelial system defense.

With the development of reliable semiquantitative mouse virulence tests, it was possible to assess the effect of growth temperatures on the virulence of the MCH76 and the CDC A2635 strains (Table 3). Virulence of both strains was not affected at all by different growth temperatures, provided that the cultures were first selected for the virulence plasmid. The undiminished virulence of *Y. enterocolitica* grown at 5°C can perhaps be one of the factors in the outbreak of yersiniosis from contaminated chocolate milk (5). The virulence of the two cultures grown at 35°C was also unchanged. This result was unexpected since invesitigators (6) had suggested that the virulence of *Y. enterocolitica* decreased when grown at 35°C. The virulence plasmid is unstable at 35°C (46), and perhaps the loss of this plasmid at this temperature was the real reason for the diminished virulence observed in the past. Extensive tests with five of the rhamnose-positive or Nilehn biotype 1 food isolates showed that these strains did not contain the virulence plasmid and were not virulent to mice by any of the following methods: oral feeding to stressed mice (unpublished results), intraperitoneal injection in saline, and intraperitoneal injection in iron dextran (Table 2). These results could not preclude the possibility that some atypical or Nilehn biotype 1 isolates could contain the virulence plasmid, unless some unknown incompatibility factor for the virulence plasmid exists among these strains. The pork isolate 78-513 and the pig's throat isolate 4-4 did contain the virulence plasmid and were as virulent to mice as the human serotype O:3 strains. In Europe, Pederson (32) and Wauters and Janssens (42) recovered the serotype O:3 strain from 20 to 50% of normal pig's throats and tongues. The virulence potential of the typical *Y. enterocolitica* isolated from pigs and pork must be regarded with caution.

In this report, it was demonstrated that the presence of a 46-Mdal plasmid in the serotype O:3 strains and a 42-Mdal plasmid in the serotype O:9 strains was associated with the properties of auto-agglutination (21), calcium dependency (16), and animal virulence (Tables 1 and 2). The virulence of these 46-Mdal plasmid-containing serotypes can be tested in Swiss mice by intraperitoneal injections in 10% iron dextran-saline solution. Infection parameters to be considered include ruffling of fur, lethargy, fecal shedding, internal liver lesions, and mortality.

The accuracy of the serotype O:3 LD_{50} determinations can be improved by selecting for more sensitive strains of mice, and also by using a

larger number of mice per dilution tested.

The results of mouse infection by plasmid-containing serotype O:9 strains in iron dextran are less conclusive, but invite further study.

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