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Pathogenicity of *Yersinia enterocolitica* Demonstrated in the Suckling Mouse

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ABSTRACT

An experimental suckling mouse intraperitoneal injection test was compared with four plasmid-associated tests (adult mouse peroral exposure, adult mouse intraperitoneal injection, autoagglutination and plasmid detection by gel electrophoresis) to measure *Yersinia enterocolitica* pathogenicity. Of eight Vwa plasmid-harboring strains (O:3; O:4,32; O:5,27; O:8; O:9; O:13; O:21; and O:Tacoma) and one isogenic plasmidless strain (O:8), all Vwa plasmid-harboring strains gave identical results in all tests except the two adult mouse tests. In studies of 35 clinical strains of *Y. enterocolitica* recently isolated during two foodborne outbreaks, a comparison of the autoagglutination, gel electrophoresis for Vwa plasmid detection and suckling mouse tests showed that 29 strains (83%) gave identical results in all three tests. The other six strains produced different reactions in the plasmid detection and autoagglutination tests, indicating that neither test alone is sufficient to evaluate the virulence of *Y. enterocolitica*. To compare the sensitivity of these in vitro tests with a biological assay (the suckling mouse intraperitoneal injection test), a mixture of plasmid-harboring (P+) and plasmidless (P−) isogenic *Y. enterocolitica* cells was examined. The suckling mouse test was more sensitive and consistent in detecting the Vwa plasmid (as evidenced by mouse lethality). A bacterial population containing 0.1% P+ cells induced a lethal infection in the suckling mouse, whereas the other two tests required at least 10% P+ cells for detection of the Vwa plasmid. The 50% lethal dose (LD_{50}) in the suckling mouse was directly proportional to the number of Vwa-harboring cells in the culture.

Human illness reportedly caused by foodborne *Yersinia enterocolitica* has been observed in Japan (*1*), Czechoslovakia (*20*), Canada (*11*) and the United States (*3,4,6,8,25*). Following the 1976 outbreak in the United States (*6*), procedures were devised for isolating *Y. enterocolitica* from foods and tests were developed for determining its pathogenicity. The rapid alkali method (*2*) was found to be useful for recovering *Y. enterocolitica* not only from foods (*4,16*) but also from animals (*9*) and humans (*3,28*).

Although strains could be recovered during foodborne outbreaks, it was not always possible to differentiate pathogenic and nonpathogenic *Y. enterocolitica* despite the use of four assays for pathogenicity: autoagglutination (*15*), 41 to 48-megadalton (Mdal) plasmid detection by gel electrophoresis (*10,30*), adult mouse peroral exposure (*15*) and adult mouse lethality (*7*). Results of in vivo and in vitro tests were not always consistent, and no clear-cut determination of pathogenicity could be made (*14,25*).

Although in vitro assays are convenient and sometimes rapid, an in vivo procedure for differentiating pathogenic and nonpathogenic strains may offer the greatest promise, at least until more is understood about the mechanisms of pathogenicity expressed by this organism. This report describes an experimental suckling mouse intraperitoneal (i.p.) injection test which appears to be useful for detecting cells that harbor plasmids associated with *Y. enterocolitica* pathogenicity. The test is especially efficacious when cultures have not been handled correctly and the Vwa plasmid has been lost from more than 90% of the cell population.

MATERIALS AND METHODS

Strains

Forty-four strains of *Y. enterocolitica* belonging to various serogroups were used in this study. The serogroup strains were obtained as follows: O:3 from Frederick Untermann, Institute for Veterinary Medicine and Life Science Hygiene, Berlin, West Germany; O:4,32; O:8 (WA+ and WA−); and O:9 from Ira J. Mehlman, Division of Microbiology, Food and Drug Administration, Washington, DC; O:21 from Eleanor Christenson, State Laboratory of Hygiene, University of Wisconsin, Madison, WI; O:8 and O:Tacoma from Dr. Carla Clausen, Children's Orthopedic Hospital, Seattle, WA, isolated from patients during the foodborne outbreak of 1981-1982 (*4*); and 35 strains of serogroup O:13 from seven hospitals in Arkansas, Mississippi and Tennessee, isolated during the foodborne outbreak of 1982 (*3*). Strain O:5,27 was isolated in our laboratory from a hog at the USDA abattoir, Beltsville, MD.

Suckling mouse i.p. injection test

Serial 10-fold dilutions of bacterial cultures grown in veal infusion broth at 22°C for 48 h were made in 0.85% saline; 0.1 ml of each dilution was injected i.p. into each of three or four 1- to 3-d-old Swiss albino mice (Sprague-Dawley ICR). The number of mice in a litter determined the number of bacterial dilutions that were tested in that litter. Injected mice were observed for 7 d. Deaths of mice within 24 h after injection were considered nonspecific. Deaths occurring between days 2 and 7 were con-

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sidered *Y. enterocolitica*-specific and were used for calculating the 50% lethal dose (LD_{50}).

Adult mouse peroral and i.p. injection tests

Adult Swiss albino mice were fed bacterial suspensions by the method of Laird and Cavanaugh (*15*). Groups of five animals, each weighing 20 to 25 g, were deprived of water for 18 to 20 h, then allowed to drink ad libitum for 24 h from a 50-ml water suspension of each of the test *Y. enterocolitica* strains grown at 22°C. Suspensions contained approximately 10^9 cells per ml. Mice were examined daily for 21 d; illnesses and deaths that occurred betweeen days 2 and 21 were considered to be induced by *Y. enterocolitica*. For comparative purposes, five other adult Swiss albino mice were given i.p. injections of a single dilution or serial 10-fold dilutions of the bacterial suspensions by a procedure identical to that used for suckling mice, except that the injected volume was 0.2 ml instead of 0.1 ml.

Autoagglutination test

This test was performed according to the method of Laird and Cavanaugh (*15*), except that isolated colonies were inoculated into two tubes containing methyl red/Voges-Proskauer (MRVP) medium (D. G. Evans, University of Texas, Houston, personal communication) instead of the Roswell Park Memorial Institute (RPMI) medium 1640 with 10% calf serum suggested by Laird and Cavanaugh. Both media gave comparable results in a preliminary autoagglutination screening test with *Y. enterocolitica* strains (data not shown). Therefore, the more plentiful and less costly MRVP medium was selected for use in this study.

Plasmid detection

Lysates were prepared from overnight cultures grown in brain heart infusion broth at 25°C. These cultures were lysed by a modification of the method developed by Kado and Liu (*12*). Lysates were incubated at 65°C for 1 h, then extracted twice with a phenol chloroform (1:1) mixture. Agarose gel electrophoresis was carried out in 0.5% gels at 125 V for 3 h in the system described by Meyers et al. (*19*). *Y. enterocolitica* strain 2383 (Lazere and Gemski, 1980, Abstr. Annu. Meet. Am. Soc. Microbiol. B11, p. 19) containing plasmids of 35 and 42 Mdal and *Escherichia coli* strain 78-IEC-1 containing plasmids of 13, 24, 44, 55, 66 and 98 Mdal (*27*) were used as standards.

Strain serogrouping

The serogroup of each strain was confirmed by the standard tube agglutination method of Winblad (*29*) with type-specific antisera prepared in New Zealand white rabbits.

RESULTS

Suckling mouse vs. adult mouse

The relative sensitivity of suckling mice (i.p. injection) and adult mice (i.p. injection and peroral exposure) to strain WA+ was determined (Table 1). After i.p. injection, the LD_{50} was 14 cells in suckling mice and 1,800 cells in adult mice. After peroral exposure in adult mice that were allowed to drink ad libitum, the LD_{50} was estimated to be greater than 6×10^6. Days of death for i.p.-injected suckling mice, i.p.-injected adult mice and perorally exposed adult mice were 1 to 5 (average 3), 2 to 19 (average 8) and 6 to 14 (average 8.7), respectively.

The effect of serogroup on the sensitivity of suckling and adult mice was determined by inoculating animals with Vwa plasmid-harboring *Y. enterocolitica* strains of serogroups O:3; O:4,32: O:5,27; O:8; O:9; O:13; O:21; and O:Tacoma (Table 2).

After peroral exposure in adult mice, five serogroup strains harboring the Vwa plasmid (O:4,32; O:8; O:13; O:21; and O:Tacoma) produced fatal infections in all test mice (days of death from 6 to 8); the remaining three serogroup strains harboring Vwa plasmid (O:3; O:5,27; and O:9) and the plasmidless O:8 strain were nonlethal. The five lethal strains were then tested in adult mice by i.p. injection. As shown in Table 2, the same five peroral-positive serogroup strains induced fatal infections in all i.p.-injected mice (days of death from 4 to 6), and the same four peroral-negative serogroup strains were nonlethal in i.p.-injected mice. In contrast, however, all eight serogroups harboring the Vwa plasmid injected i.p. into suckling mice induced fatal infections, with deaths occurring from days 2 to 5. The LD_{50} among these serogroup strains ranged from 10 to 10,000 cells. The O:8 strain cured of its plasmid was nonlethal even when 3×10^6 cells were injected.

Suckling mouse vs. Vwa plasmid detection tests

Two conventional methods for detecting Vwa plasmids were compared with the new suckling mouse i.p. injection test (Table 3). Of the 35 clinical strains tested, 23 gave positive results in all tests, six gave negative results in all tests and the other six gave variable results. Four strains were positive in two of three tests and two were positive in only one test. The mouse LD_{50} for the virulent cultures varied from 50 to 60,000 cells.

TABLE 1. *Titration of cultured* Y. enterocolitica *(strain WA, serotype O:8) in suckling and adult mice.*

	Suckling mouse		Adult mouse			
	I.p. injection (0.1 ml)		Peroral route (ad libitum)		I.p. injection route (0.2 ml)	
WA suspension Cells/ml	No. dead/ injected	Day of death (No. dead)	No. dead/ exposed	Day of death (No. dead)	No. dead/ injected	Day of death (No. dead)
2×10^8	8/8	1(6),2(2)	5/5	6(2),7(2),9(1)	5/5	2(1),3(4)
2×10^7	9/9	2(6),3(3)	4/5	8(1),9(2),12(1)	5/5	4(3),6(2)
2×10^6	9/9	2(4),3(5)	1/5	15(1)	5/5	5(1),6(2),8(2)
2×10^5	10/10	2(6),3(4)	0/5		5/5	5(1),7(3),10(1)
2×10^4	7/7	2(3),3(2),4(2)	0/5		3/5	7(1),16(1),19(1)
2×10^3	8/8	3(3),4(3),5(2)	0/5		4/5	10(1),16(2),19(1)
2×10^2	7/7	3(3),4(3),5(1)	0/5		1/5	15(1)
2×10^1	10/12	3(4),4(5),5(1)	0/5		0/5	
2×10^0	0/9		0/5		0/5	

Sensitivity of Vwa plasmid detection tests

A comparison was made of the sensitivity of the gel electrophoresis, autoagglutination and suckling mouse i.p. injection tests for detecting the Vwa plasmid in an O:3 strain of *Y. enterocolitica*. Brain heart infusion cultures of isogenic strains, one harboring the Vwa plasmid (P+) and the other spontaneously cured of this plasmid (P−), were mixed and immediately examined for the presence of the Vwa plasmid by gel electrophoresis and by the suckling mouse injection method. The mixtures were also streaked to trypticase soy agar plates and incubated at 22°C for 48 h. Twenty colonies from each plate were then examined for presence of the Vwa plasmid by the autoagglutination method. Findings with mixtures of P+ and P− cells in the three plasmid-associated tests are presented in Table 4.

All P+ cell mixtures induced fatal infections in suckling mice, with the LD_{50} ranging from 2×10^3 to 2×10^6 cells. The bacterial suspension containing only P− cells was not lethal for suckling mice. The Vwa plasmid was detected by all three methods in mixtures containing more than 10% P+ cells. Mixtures containing less than 10% P+ cells gave negative results in both the gel electrophoresis and autoagglutination tests, but positive results in the suckling mouse i.p. injection test.

DISCUSSION

Recent *Y. enterocolitica* infections in humans (*1,3-8,20*) demonstrate the need for a sensitive and dependable test for the pathogenicity of this bacterial species. Zink (*30*) re

TABLE 2. *Mortality in suckling and adult mice with eight selected* Y. enterocolitica *serotypes.*

Serotype	Source	Plasmid size (Mdal)	Autoagglutination (37°C)	Suckling mouse i.p. injection (LD_{50})	Adult mouse Peroral route (ad libitum)[a] No. dead/No. exposed	Adult mouse I.p. injection[b] No. dead/No. injected
O:3	Pig tongue	44	+	1×10^3	0/5	0/5
O:4,32	Human	42	+	5×10^1	5/5	5/5
O:5,27	Pig tongue	42	+	3×10^3	0/5	0/5
O:8	Human	42	+	1×10^1	5/5	5/5
O:9	Human	42	+	1×10^4	0/5	0/5
O:13	Human	42	+	2×10^1	5/5	5/5
O:21	Human	42	+	5×10^1	5/5	5/5
O:Tacoma	Human	42	+	5×10^1	5/5	5/5
O:8	Human	None	−	Nonlethal[c]	0/5	0/5

[a]Inoculum contained 10^9 cells/ml. Mice drank ad libitum for 24 h.
[b]Inoculum contained 2×10^6 cells.
[c]3×10^6 cells injected i.p.

TABLE 3. *Results of three plasmid-associated pathogenicity tests with 35 clinical isolates of* Y. enterocolitica.

No. of strains (Serotype)	Auto-agglutination (22°C/37°C)	Gel electro-phoresis	Suckling mouse i.p. injection (LD_{50})
21(O:13),1(O:8),1(O:Tacoma)	−/+	+	50-1,000
3(O:13)	−/+	−	60,000
1(O:13)	−/−	+	100
1(O:13	−/+	−	Nonlethal[a]
1(O:Tacoma)	−/−	+	Nonlethal[a]
3(O:13),3(O:8)	−/−	−	Nonlethal[a]
No. positive/No. examined	27/35	25/35	27/35
Percent positive	77	71	77

[a]3×10^6 cells injected i.p.

TABLE 4. *Sensitivity of three VWa plasmid detection tests to* Y. enterocolitica *strain O:3.*

P+ cells (%)	Autoagglutination (37°C) No. of colonies positive/ No. of colonies tested	Gel electro-phoresis	Suckling mouse i.p. test (LD_{50})
0	0/20	None	nonlethal
100	20/20	44 Mdal	2×10^{3a}
10	1/20	±[b]	1×10^{4a}
1	0/20	None	1×10^{5a}
0.1	0/20	None	2×10^{6a}

[a]P+ cells were recovered from livers and spleens of dead mice.
[b]A faint 44-Mdal plasmid band was observed.

ported that a plasmid (41 to 48 Mdal) was responsible for virulence; plasmids of 42 to 48 Mdal have been found in strains associated with human disease. Kay et al. (*13*) reported that a plasmid (82 Mdal) was responsible for virulence and Laird and Cavanaugh (*15*) proposed an autoagglutination test for the rapid detection of the Vwa plasmid. Some investigators have shown 100% correlation between autoagglutination results and findings in either the gel electrophoresis or mouse diarrheal tests (*15,17,23,26*). Other investigators who used the same autoagglutination medium (RPMI-1640) as those who reported 100% correlation in the autoagglutination test have reported inconsistent findings in these tests (*4,14,18,21,25*). These conflicting reports led us to compare a suckling mouse i.p. injection test with four virulence tests currently in use for detecting the Vwa plasmid in *Y. enterocolitica*.

Whole animal tests which use adult mice to detect pathogenic strains of *Y. enterocolitica* have often relied on the induction of diarrhea as an end point (*15,23,24*); however, in mice diarrhea may be difficult to define and is not easily quantitated (*21*). A mouse-holding method described by Schiemann (*23*) and Schiemann et al. (*24*) for collecting test mouse feces and comparing them with normal mouse feces (for shape, consistency, moisture and mucous content) is dependent upon the subjective interpretation of the investigator. Therefore, a more clear-cut end point (death) was used in this study.

Table 1 shows that compared with adult mice, suckling mice were about 100 times more sensitive to infection with plasmid-harboring cells, and the mean day of death occurred 11 days earlier. Therefore, the suckling mouse was killed by fewer cells in less time than was the adult mouse.

The effect of *Y. enterocolitica* serogroups on suckling and adult mouse lethality is presented in Table 2. Cells of *Y. enterocolitica* serogroups O:3; O:4,32; O:5,27; O:8; O:9; O:13; O:21; and O:Tacoma injected i.p. into suckling mice were lethal, with the mean day of death approximately 2 to 3 d. Strains of serogroups O:3; O:5,27; and O:9 injected into adult mice were not lethal, but serogroups O:4,32; O:8; O:13; O:21; and O:Tacoma killed adult mice, with day 5 as the average day of death. Adult mice exposed by the peroral route were not killed by serogroup strains O:3; O:5,27; and O:9 but were killed by serogroups O:4,32; O:8; O:13; O:21; and O:Tacoma, with day 7 as the average day of death. Interestingly, strains that were lethal for adult mice also required fewer cells to kill 50% of the suckling mice. It was later determined that in the adult mouse, lethal strains elaborated lipase and nonlethal strains did not (*5*).

It was postulated that lipase may destroy the lipid barriers and permit bacteria to invade neighboring tissues to produce a fulminating lethal infection. Similarly, it was found (Fanning, G. R., J. R. Lazere, J. N. Coulby, J. A. Wohlheiter, and P. Gemski, 1983, Abstr. Annu. Meet. Am. Soc. Microbiol., B78, p. 36) that certain serotype O:8 strains of *Y. enterocolitica* harbored both the Vwa and 82-Mdal plasmids and produced disseminating lethal infections in adult mice; the serotype O:3 strain that harbored only the Vwa plasmid produced only a local infection in the gut. The lysis method of Kado and Liu (*12*) did not reveal the 82-Mdal plasmid in any of the 35 *Y. enterocolitica* strains tested (Table 3). However, with a modified procedure (Fanning et al., cited above), we were able to demonstrate the 82-Mdal plasmid in 22 of 27 lethal strains. Although the nonlethal O:Tacoma strain harbored a 42-Mdal plasmid, the presence of an 82-Mdal plasmid could not be demonstrated.

Findings with eight selected serogroup strains of *Y. enterocolitica* that harbor the Vwa plasmid showed a 100% correlation in the gel electrophoresis, autoagglutination and suckling mouse lethality tests (Table 2). However, when 35 freshly isolated clinical strains were used, agreement among tests was only 83% (Table 3). Some strains gave positive results in two tests but not in the third, and some were positive in one test but not in the other two. Agreement between the suckling mouse test results and the gel electrophoresis and autoagglutination tests with freshly isolated clinical strains was 90 and 94%, respectively. Agreement between the gel electrophoresis and autoagglutination tests was 83%. All strains positive in the suckling mouse test were also positive in either one or both of the other tests; however, a positive reaction in either the gel electrophoresis or autoagglutination tests was sometimes accompanied by negative reactions in the other two tests. Both Mehlman (*18*) and Lee (*17*) experienced irregularities in the autoagglutination test. Other investigators (*14,25*) have recently reported no correlation among plasmid-associated in vitro and in vivo tests and have indicated the need for an animal model.

In our study, one strain (O:Tacoma), which carried the 42- but not the 82-Mdal plasmid, gave negative results in the suckling mouse and autoagglutination tests. An earlier observation with an O:3 food isolate gave similar reations in these tests as did the exceptional O:Tacoma strain (*4*). Previous reports have shown the presence of a 42-Mdal plasmid in an avirulent *Y. enterocolitica* strain (*13,22*). Colony hybridization tests indicated that the O:3 strain did not contain the nucleotide sequences of the calcium dependence region of plasmid pYV8081 (data not shown; *13,22*).

The reactions of five of six O:13 strains (Table 3) cannot be explained as easily as those with the O:Tacoma strain. Three O:13 cultures produced positive results in the autoagglutination and suckling mouse tests and negative results in the electrophoresis tests. These cultures may have contained fewer Vwa plasmid-harboring cells, which could not be detected by the gel electrophoresis method but could be detected by the suckling mouse test and by chance selection in the autoagglutination tests. This hypothesis is supported by several facts. For example, the suckling mouse LD_{50} for these cultures was 60,000 cells, whereas similar virulent serotype cultures produced an LD_{50} of 50 to 1,000 cells. Also, a positive autoagglutination test usually depends upon the chance selection of a P+ colony. The fact that the autoagglutination results for the two questionable strains differed from those obtained in the suckling mouse

and electrophoresis tests suggests inherent problems in the autoagglutination test (*17,18*).

Recently, it was reported that findings with 17 of 100 *Y. enterocolitica* strains in the autoagglutination and calcium dependency tests were not consistent (*14*). Results of either test usually predict those of the other; however, if the end point in the autoagglutination test is not clear-cut, the findings are dependent upon interpretation. The suckling mouse lethality test has a clear-cut end point (death), and the animal is susceptible to all serogroup *Y. enterocolitica* strains harboring the Vwa plasmid (*5*). These two characteristics have never been observed in other animal models.

The observation that 8 of 35 *Y. enterocolitica* strains of human origin were not lethal in suckling mice deserves comment. It has been reported (*22*) that in some instances *Y. enterocolitica* plasmid-associated virulence factors are lost by multiple serial transfers of cultures and in other instances by incubation of cultures at temperatures above 35°C. All 35 strains recovered from humans were isolated at 37°C and transferred two or three times before being received by our laboratory. This excessive handling may have caused plasmid loss, thereby limiting the usefulness of these tests.

The sensitivity of the three plasmid-associated tests was evaluated with bacterial suspensions containing a mixture of P+ and P− cells. Our findings (Table 4) showed that the suckling mouse test was at least 100 times more sensitive than the gel electrophoresis and autoagglutination tests used in examining mixed cultures. The Vwa plasmid was detected in the suckling mouse test when the bacterial population consisted of only 0.1% P+ cells (the lowest concentration of P+ cells tested). In comparison, detection of the Vwa plasmid in the gel electrophoresis and autoagglutination tests requires that at least 10% of the bacterial cell population be positive. In addition, the LD_{50} value for the bacterial mixtures in suckling mice is directly proportional to the number of P+ cells in the bacterial suspension. All strains recovered from spleens and livers of dead mice after inoculation with mixtures of P+ and P− cells were shown to have the Vwa plasmid and to autoagglutinate at 37°C (data not shown).

Our data strongly suggest that the Vwa plasmid is associated with lethality in the suckling mouse and that positive or negative reactions in the gel electrophoresis and autoagglutination tests may sometimes misrepresent the true pathogenic status of the organism. The suckling mouse i.p. injection test appears to be more sensitive and dependable than either the gel electrophoresis or autoagglutination tests for detection of *Y. enterocolitica* strains that harbor Vwa plasmid. Plasmidless cells are nonlethal for suckling mice and may also be nonpathogenic for humans. Furthermore, the suckling mouse i.p. injection test appears to be a more accurate measure of pathogenesis, i.e., the bacterial cells are injected into a viable environment which more closely resembles that in which they exist during human infection.

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REFERENCES

1. Asakawa, Y., S. Akahane, N. Kagata, M. Noguchi, R. Sakazaki, and K. Tamura. 1973. Two community outbreaks of human infection with *Yersinia enterocolitica*. J. Hyg. 71:715-723.
2. Aulisio, C. C. G., I. J. Mehlman, and A. C. Sanders. 1980. Alkali method for rapid recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from foods. Appl. Environ. Microbiol. 39:135-140.
3. Aulisio, C. C. G., J. M. Lanier, and M. A. Chappel. 1982. *Yersinia enterocolitica* O:13 associated with outbreaks in three southern states. J. Food Prot. 45:1263.
4. Aulisio, C. C. G., J. T. Stanfield, S. D. Weagant, and W. E. Hill. 1983. Yersiniosis associated with tofu consumption: serological biochemical and pathogenicity studies of *Yersinia enterocolitica* isolates. J. Food Prot. 46:226-230.
5. Aulisio, C. C. G., W. E. Hill, J. T. Stanfield, and R. L. Sellers, Jr. 1983. Evaluation of virulence factor testing and characteristics of pathogenicity in *Yersinia enterocolitica*. Infect. Immun. 40:330-335.
6. Black, R. E., R. J. Jackson, T. Tsai, M. Medvesky, M. Shayegani, J. C. Feeley, K. I. E. MacLeod, and A. M. Wakelee. 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. N. Engl. J. Med. 298:76-79.
7. Carter, P. B., and F. M. Collins. 1974. Experimental *Yersinia enterocolitica* infection in mice: kinetics of growth. Infect. Immun. 9:851-857.
8. Centers for Disease Control. 1982. Multi-state outbreak of yersiniosis. Morbid. Mortal. Weekly Rep. 31:505-506.
9. Doyle, M. P., M. B. Hugdahl, and S. L. Taylor. 1981. Isolation of virulent *Yersinia enterocolitica* from porcine tongues. Appl. Environ. Microbiol. 42:661-666.
10. Gemski, P., J. R. Lazere, and T. Casey. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. 27:682-685.
11. Health and Welfare Canada. 1976. *Yersinia enterocolitica* gastroenteritis outbreak - Montreal. Can. Dis. Weekly Rep. 2:73-74.
12. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.
13. Kay, B. A., K. Wachsmuth, and P. Gemski. 1982. New virulence-associated plasmid in *Yersinia enterocolitica*. J. Clin. Microbiol. 15:1161-1163.
14. Kay, B. A., K. Wachsmuth, P. Gemski, J. C. Feeley, T. J. Quan, and D. J. Brenner. 1983. Virulence and phenotypic characterization of *Yersinia enterocolitica* isolated from humans in the United States. J. Clin. Microbiol. 17:128-138.
15. Laird, W. J., and D. C. Cavanaugh. 1980. Correlation of autoagglutination and virulence of *Yersinia*. J. Clin. Microbiol. 11:430-432.
16. Lee, W. H., M. E. Harris, D. McClain, R. E. Smith, and R. W. Johnston. 1980. Two modified selenite media for the recovery of *Yersinia enterocolitica* from meats. Appl. Environ. Microbiol. 39:205-209.
17. Lee, W. H., R. E. Smith, J. M. Damare, M. E. Harris, and R. W. Johnston. 1981. Evaluation of virulence test procedures for *Yersinia enterocolitica* recovered from foods. J. Appl. Bacteriol. 50:529-539.
18. Mehlman, I. J., C. C. G. Aulisio, A. Romero, and A. C. Sanders. 1981. Recovery of potentially enteropathogenic *Yersinia* species from food and clinical specimens. pp. 412-424. *In* T. A. Roberts, G. Hobbs, J. H. B. Christian, and N. Skovgaard (eds.) Psychrotrophic microorganisms in spoilage and pathogenicity. Academic Press, London.
19. Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and char-

con't. p. 863

The present study used high protein foods. It remains to be seen if the presence of urea in the extracting fluid influences the recovery of botulinum toxin from foods of other compositions.

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REFERENCES

1. Dowell, V. R., and T. M. Dawkins. 1968. Laboratory methods in anaerobic bacteriology. *In* CDC laboratory manual. U.S. Government Printing Office, Washington, DC.
2. Duff, J. T., G. G. Wright, J. Klerer, D. E. Moore, and R. H. Bibler. 1957. Studies on immunity to toxins of *Clostridium botulinum* I. A simplified procedure for isolation of type A toxin. J. Bacteriol. 73:42-47.
3. Hustad, G. O., J. G. Cerveny, H. Trenk, R. H. Deibel, D. A. Kautter, T. Fazio, R. W. Johnston, and O. E. Kolari. 1973. Effect of sodium nitrite and sodium nitrate on botulinal toxin production and nitrosamine formation in wieners. Appl. Microbiol. 26:22-26.
4. Kautter, D. A., and R. K. Lynt. 1976. *Clostridium botulinum*. pp. 424-436. *In* M. L. Speck (ed.) Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, DC.
5. Kautter, D. A., and R. K. Lynt. 1978. *Clostridium botulinum*. Chapter XIV, pp. 1-16. *In* Food and Drug Administration, Bacteriological analytical manual. 5th ed. Association of Official Analytical Chemists, Washington, DC.
6. Kozaki, S., S. Togashi, and G. Sakaguchi. 1981. Separation of *Clostridium botulinum* type A derivative toxin into two fragments. Jap. J. Med. Sci. Biol. 34:61-68.
7. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.
8. Tanaka, N., E. Traisman, M. H. Lee, R. G. Cassens, and E. M. Foster. 1980. Inhibition of botulinum toxin formation in bacon by acid development. J. Food Prot. 43:450-457.
9. Wang, H. L., E. W. Swain, and C. W. Hesseltine. 1975. Mass production of *Rhizopus oligosporus* spores and their application in tempeh fermentation. J. Food Sci. 40:168-170.
10. Wang, H. L., and C. W. Hesseltine. 1982. Coagulation conditions in tofu processing. Process Biochem. 17:7-12.

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acterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.
20. Olsousky, Z., S. Chobot, and V. Sviridov. 1975. Mass occurrence of *Yersinia enterocolitica* in two establishments of collective care of children. J. Hyg. Epidemiol. Microbiol. Immunol. 19:22-29.
21. Pai, C. H., and L. DeStephano. 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. 35:605-611.
22. Portnoy, D. A., S. L. Moseley, and S. Falkow. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. 31:775-782.
23. Schiemann, D. A. 1981. An enterotoxin-negative strain of *Yersinia enterocolitica* serotype O:3 is capable of producing diarrhea in mice. Infect. Immun. 32:571-574.
24. Scheimann, D. A., J. A. Devenish, and S. Toma. 1981. Characteristics of virulence in human isolates of *Yersinia enterocolitica*. Infect. Immun. 32:402-403.
25. Shayegani, M., D. Morse, I. DeForge, T. Root, L. M. Parsons, and P. S. Maupin. 1983. Microbiology of a major foodborne outbreak of gastroenteritis caused by *Yersinia enterocolitica* serogroups O:8. J. Clin. Microbiol. 17:35-40.
26. Stern, N. J., and J. M. Damare. 1982. Comparison of selected *Yersinia enterocolitica* indicator tests for potential virulence. J. Food Sci. 47:582-588.
27. Stieglitz, H., R. Fonseca, J. Olarte, and Y. M. Kuperztoch-Portnoy. 1980. Linkage of heat-stable enterotoxin activity and ampicillin resistance in a plasmid isolated from an *Escherichia coli* strain of human origin. Infect. Immun. 30:617-620.
28. Weissfeld, A. S., and A. C. Sonnenwirth. 1982. Rapid isolation of *Yersinia* spp. from feces. J. Clin. Microbiol. 15:508-510.
29. Winblad, S., B. Nielhn, and N. H. Sternby. 1966. *Yersinia enterocolitica* (Pasteurella X) in human enteric infections. Br. Med. J. 2:1363-1366.
30. Zink, D. L., J. C. Feeley, J. G. Wells, C. Vanderzant, J. C. Vickery, W. D. Roof, and G. A. O'Donovan. 1980. Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature 283:224-226.