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Serum Resistance of *Yersinia enterocolitica* Expressed in Absence of Other Virulence Markers

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Serum resistance of *Yersinia enterocolitica* after growth at 37 and 25°C appears to be specific for serogroup O:3 and appears to be expressed even in the absence of other phenotypic virulence-associated markers, such as the presence of V antigen, autoagglutination, and calcium dependency after growth at 37°C.

The clinical manifestations of *Yersinia enterocolitica* infections (e.g., gastroenteritis, mesenteric lymphadenitis, terminal ileitis, and septicemia) have long been related to the nature of the host response. Microbiologically, however, virulence of *Y. enterocolitica* has been a function of the serogroup and biotype of the infecting strain (8), the presence of the VW antigen complex (1), and plasmids which have been correlated with the expression of in vivo and in vitro virulence factors (2, 5, 7, 10, 11).

The major in vivo models for determining virulence have involved lethality for laboratory animals upon intraperitoneal and oral administration (1, 2) and keratoconjunctivitis (9) upon the instillation into the eyes of guinea pigs. In vitro tests have included HeLa cell invasiveness (infectivity) (8), autoagglutination (5), and calcium dependency for growth at 37°C (3). The latter two characteristics have been found in association with the presence of the VW antigen complex (1, 2). Recently, Pai and DeStephano (6) have shown that serum resistance is also associated with virulence in *Y. enterocolitica* and that, analagous to the other virulence markers (e.g., calcium dependency, autoagglutination, and production of V and W antigens), serum resistance is a temperature-dependent phenomenon expressed by cells after growth at 37 but not at 25°C. Pai and DeStephano (6) have shown that serum resistance is only demonstrated among strains that are virulent for rabbits and mice and that manifest calcium dependency and autoagglutination at 37°C. Isogenic mutants lacking these virulence markers are susceptible to serum bactericidal activity even after growth at 37°C.

While studying the virulence factors associated with 22 recent human isolates of *Y. enterocolitica*, we encountered 11 strains which lacked the virulence-associated properties of calcium dependency and autoagglutination but were nevertheless serum resistant.

The 22 *Y. enterocolitica* strains studied, derived from patients residing within a 25-mile radius of New York City, were characterized through standard methods and were serogrouped through the courtesy of S. Toma, Ministry of Health, Toronto, Canada. Four of the earlier isolates from serogroups O:3, O:5,27, O:8, and O:9 served as antigens for serological studies and were considered stock strains, having been subcultured to Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) at weekly intervals at 25°C since 1978. The more recent strains had undergone a maximum of three or four subcultures on sheep blood agar at 25°C before testing. In addition to *Y. enterocolitica*, one strain of *Y. intermedia* was also included.

As shown in Table 1, 11 *Y. enterocolitica* isolates that autoagglutinated at 37°C and showed a requirement for calcium as evidenced by a failure to grow on magnesium oxalate agar (MOX) at 37°C (2, 3) were serum resistant after growth at 37°C as determined by the method of Pai and DeStephano (6). None of these strains, comprising serogroups O:3, O:5,27, and O:8, showed a significant decrease in colony-forming units (CFU) after exposure for 2 h of 10^7 cells per ml to 10% (vol/vol) pooled human serum in Hanks balanced salt solution with 0.1% gelatin. Counts of viable organisms in these instances were equivalent to those obtained in the absence of serum. These strains also retained autoagglutination and calcium dependency during the 2 h of serum exposure. Serum resistance among the eight serogroup O:3 isolates, in contrast to the results of Pai and DeStephano (6) with their serogroup O:3 isolate, was even manifested after growth of these test strains at 25°C. In addition,

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TABLE 1. Serum resistance of autoagglutination-positive calcium-dependent strains of *Y. enterocolitica*

Strain no.[a]	Sero-group	Bio-type[b]	Source	Viability count (log_{10} CFU/ml)[c]			
				60 min		120 min	
				37°C	25°C	37°C	25°C
2	O:3	4	Stool	6.34	5.19	6.34	5.15
3	O:3	4	Stool	6.43	5.86	6.40	6.16
6	O:3	4	Stool	6.73	6.33	6.74	6.14
5	O:3	4	Stool	6.62	6.28	6.73	5.95
10	O:3	4	Stool	6.81	6.50	6.80	6.33
9	O:3	4	Stool	6.95	6.93	7.02	6.83
17	O:3	4	Stool	7.08	6.37	6.95	6.93
18	O:3	4	Stool	6.82	5.25	6.72	5.06
13	O:8	1	Appendix	6.56	4.49	6.46	3.32
8	O:8	1	Stool	6.27	2.11	6.43	1.90
14	O:5, 27	2	Blood	6.95	3.36	6.54	1.30

[a] All strains listed autoagglutinated at 37°C. In tests made before and during serum exposure, all grew on MOX agar at 25 but not at 37°C.
[b] According to G. Wauters (Ph.D. thesis, University, 1970).
[c] Average count at time zero, 6.71. Pooled human serum was stored at −70°C in 5-ml portions until used; it was then discarded.

two serogroup O:3 variants (numbers 9 and 17) which had been cured of their 41-megadalton (41-Mdal) plasmid DNA also showed serum resistance after growth at 25 and 37°C (Table 2). Neither the two parent strains nor their cured isogenic derivatives were lethal for 6- to 8-week-old white mice upon intraperitoneal injection of 10^7 CFU/ml. Only the two serogroup O:8 isolates (numbers 13 and 8) and the serogroup O:5,27 isolate (number 14), both of which were lethal for white mice, showed serum sensitivity after growth at 25°C. Our findings on the serum sensitivity of these latter serogroups parallels those of Pai and DeStephano with their strains from serogroups O:5,27, O:1,2,3 and O:6:30.

Surprisingly, serum resistance after growth at 37°C was noted among 9 of 11 *Y. enterocolitica* isolates, including the four stock strains, which lacked the virulence-associated phenotypic markers of autoagglutination and calcium dependency at 37°C. None of these nine strains, comprising representatives of serogroups O:3, O:5,27, O:8, and O:9, showed a significant decrease in CFU after growth at 37°C and serum exposure for 2 h (Table 3). Serologically, seven of these isolates forwarded to R. R. Brubaker, Michigan State University, East Lansing, Mich., were shown to lack V antigen; this correlated with their lack of calcium dependency and autoagglutination (2). Four representatives of these seven strains (numbers 1, 7, 15, and 19) lacked mouse lethality. Only two strains, both serogroup O:5,27, one blood and one stock strain, showed relative serum susceptibility after growth at 37°C as determined by drops of three and four logs, respectively, in CFU after 2 h of incubation in serum. None of the 11 strains autoagglutinated and all maintained the ability to grow on MOX agar after exposure to human serum for 2 h, thereby confirming a lack of reversion to virulence (calcium dependency) during incubation. After growth at 25°C, most of

TABLE 2. Serum resistance of two isogenic mutants of serogroup O:3 *Y. enterocolitica* cured of 41-Mdal plasmid species

Strain no.[a]	Plasmid species	Autoagglutination at 37°C	Growth on MOX agar at 37°C[b]	Viability count (log_{10} CFU/ml)[c]			
				60 min		120 min	
				37°C	25°C	37°C	25°C
9	41 Mdal	+	0	6.64	6.23	6.56	7.23
9A	Cured	0	+	6.82	6.30	5.80	7.07
17	41 Mdal	+	0	7.00	6.93	6.00	7.07
17A	Cured	0	+	6.92	6.80	6.14	6.94

[a] All strains listed were serogroup O:3, biotype 4 (biotype according to Wauters, Ph.D. thesis). None proved lethal to 6- to 8-week-old mice inoculated with 10^7 CFU/ml and observed for 21 days.
[b] Results obtained before and during serum exposure.
[c] Average count at time zero, 6.71.

TABLE 3. Serum resistance of autoagglutination-negative non-calcium-dependent yersiniae

Strain[a]	Sero-group	Bio-type[b]	Source	V antigen	Viability count (log_{10} CFU/ml)[c]			
					60 min		120 min	
					37°C	25°C	37°C	25°C
Y. entero-colitica								
19	O:3	4	Blood	0	6.75	5.15	6.59	3.14
7	O:8	1	Stool	0	6.76	2.71	6.62	1
12	O:8	1	Abscess	0	6.74	5.47	6.52	2.94
28	O:8	1	Stool	0	6.23	5.49	6.02	5.24
1	O:5, 27	2	Blood	0	6.45	5.77	2.67	3.02
15	O:5, 27	2	Stool	0	6.51	5.36	6.66	2.36
16	O:5, 27	2	Stool	0	6.52	4.22	6.36	2.47
A	O:3	4	Stock	ND[d]	6.61	6.66	6.65	5.13
B	O:5, 27	2	Stock	ND	5.14	6.38	3.35	2.99
C	O:8	1	Stock	ND	6.75	6.38	6.68	5.34
D	O:9	3	Stock	ND	6.39	6.39	6.32	4.26
Y. intermedia	O:17	1	Stock	ND	3.07	5.91	2.07	2.86

[a] All strains listed autoagglutinated at 37°C. In tests made before and during serum exposure, all grew on MOX agar at 37°C.
[b] According to Wauters (Ph.D. thesis).
[c] Average count at time zero, 6.60.
[c] ND, Not done.

these strains showed a moderate susceptibility to the bactericidal action of normal human serum within 2 h. The *Y. intermedia* strain was inhibited by human serum within 60 min irrespective of the temperature of growth. Heat inactivation (56°C, 1 h) of serum abolished bactericidal activity of all 23 strains irrespective of growth temperature.

The results of our study indicate that temperature-related serum resistance of *Y. enterocolitica* may well be expressed in the absence of other virulence markers associated with the presence of a plasmid species (2, 7). Our serogroup O:3 isolates which expressed calcium dependency and autoagglutination resisted serum bactericidal activity irrespective of growth temperature before testing. This was true even of two isogenic mutants cured of their 41-Mdal plasmid, which are known to be associated at 37°C with calcium dependency, autoagglutination, and the synthesis of the VW antigen complex. The two serogroup O:8 isolates (numbers 13 and 8) and the O:5,27 isolate (number 14) did show the temperature-related serum bactericidal effect which correlated with autoagglutination and calcium dependency. It was found, however, that *Y. enterocolitica* isolates of pathogenic serogroups (O:3, O:8, O:5,27, O:9) which were negative for autoagglutination, calcium dependency, mouse virulence (O:3, O:5,27, O:8), and V antigen, and which therefore were regarded as avirulent, also expressed serum resistance after growth at 37°C. This finding supports the concept that serum resistance is an independent marker. It may be that this feature is encoded by a plasmid distinct from the 41-Mdal plasmid which is associated with the phenotypic expression at 37°C of autoagglutination, calcium dependency, mouse virulence (O:8), and the VW antigen complex. Our data for the two isogenic mutants of serogroup O:3, which lack the 41-Mdal plasmid, and the serum resistance of the 11 *Y. enterocolitica* strains that lack the virulence markers associated with the 41-Mdal plasmid corroborate the role for a separate entity accounting for serum resistance. Recently, Kay and colleagues (4) have described a heretofore undetected 82-Mdal plasmid associated with *Y. enterocolitica* mouse virulence. Perhaps a similar or different determinant may account for serum resistance in *Y. enterocolitica* by coding for another capsular antigen distinct from VW.

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