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Comparison of Three Tests for Virulent *Yersinia enterocolitica*

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Magnesium oxalate agar was found to select against virulent types of *Yersinia enterocolitica*. Small colonies isolated from magnesium oxalate agar which were presumably virulent generally contained no detectable virulence-specific plasmids, did not agglutinate with virulence-specific antiserum, and exhibited various degrees of virulence in mice. Results indicate that the virulence potential of a *Y. enterocolitica* isolate cannot always be identified by these in vitro methods and suggest that virulence also involves other factors yet to be identified.

Yersinia enterocolitica causes a variety of infections in humans, including acute gastroenteritis, mesenteric adenitis, septicemia, arthritis, and erythema nodosum (4, 5, 22). The presence of the 42- or the 82-megadalton or both plasmids is associated with numerous virulence traits of this organism (9, 10, 13, 21, 23, 24). These traits include mouse lethality (20), autoagglutination (12, 14), production of V (immunogenic protein) and W (nonprotective lipoprotein) antigens (6, 17), serum resistance (16), calcium dependency (1, 6, 9), production of specific outer membrane polypeptides (3, 7, 15, 18), restricted growth on Congo red agar (19), and agglutination reaction with specific antiserum (WA-SAA) prepared against a virulent strain (WA) of *Y. enterocolitica* (8).

In laboratory subcultures we have often encountered the loss of virulence traits from originally virulent isolates of *Y. enterocolitica*. We have therefore examined methods reported to selectively differentiate virulent from avirulent isolates of *Y. enterocolitica*. One method is based on the use of magnesium oxalate agar (MOX; 11), which has been reported to differentiate virulent and avirulent cultures of *Yersinia pestis* (11) as well as *Y. enterocolitica* (6, 9). When grown at 37°C, virulent isolates appear as small colonies on MOX because of calcium dependency, whereas avirulent isolates appear as large colonies. Two other methods were evaluated along with the MOX selection assay. These included examining isolates for a virulence-associated plasmid profile and determining the agglutination reaction of the organism with WA-SAA. We have shown previously that WA-SAA agglutinates specifically with virulent isolates of *Y. enterocolitica* that cause diarrhea or death in mice (8) and have recently shown that the agglutination reaction correlates with the presence of the virulence plasmids and the production of specific temperature-dependent outer membrane polypeptides (7).

Using established avirulent isolates of serotype O:8 (WA-ETBR, Y7N, and FRI-A2635N; 8), we observed only uniformly large-size colonies on MOX. However, virulent isolates from several serotypes, including O:3, O:4,32, O:5,27, O:8, O:9, and O:21, produced both large and small colonies on MOX. The large colonies were shown previously to be plasmidless, did not produce the specific outer membrane polypeptides, and did not agglutinate with WA-SAA (7). The small colonies were assumed to represent virulent derivatives and were examined extensively in this study. Strain WA (serotype O:8) was used in most of this study because it has been used commonly by us and others as a reference strain of pathogenic *Y. enterocolitica* and was also used for the production of WA-SAA.

First, we examined the correlation of the preponderance of small colonies with the presence of virulence plasmids and with agglutination titers with WA-SAA (Table 1). The percentage of small colonies on MOX varied from 3 to 81% in various cultures of strain WA from different laboratory passages. Plasmids were detected in all cultures with high proportions of small colonies (≥50%) and in four of five cultures with an intermediate proportion (20 to 35%) but were not detected in cultures with a very low proportion of small colonies (3 to 4%). We then examined whether the small colonies represent virulent, plasmid-bearing cells and whether a decrease in the proportion of small colonies is related to a reduction in the percentage of cells containing plasmids, therefore reducing the chance of plasmid detection.

Small colonies of strain WA were isolated from the MOX plates, cultured overnight at 37°C in Trypticase soy broth (TSB; BBL Microbiology Systems), and examined for agglutination titers and plasmid content. Of 55 small colonies randomly picked in four separate experiments, 54 did not possess either virulence plasmid and did not agglutinate with WA-SAA (titer, <240). The one virulence plasmid-positive colony autoagglutinated in the microtiter assay, hence its ability to agglutinate with WA-SAA could not be determined. We also found a low incidence of plasmid-containing cultures from MOX-grown small-colony isolates of strains E675 and C122-76 (both, serotype O:3) and strain IP-383 (serotype O:9). This high frequency of virulence plasmidless small colonies was unexpected. The results suggest that either MOX or the subsequent subculture in TSB select against plasmid-containing cells. We doubt that TSB selects against plasmid replication because in our experience the virulence plasmids of TSB-grown cultures generally exhibit stronger banding intensity on agarose gels than do the same plasmids from cultures grown on other conventional media, such as brain heart infusion broth. Also, cultures grown in TSB and maintained refrigerated in the same medium for 2 to 3 weeks retained their virulence plasmids. It is likely that MOX is selective against cells containing virulence plasmids. We have observed that the small MOX-grown colonies are pinpoint after 24 h, attain a diameter of 1 mm after 40 to 48 h (the large colonies are 1 mm in diameter after 24 h and

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TABLE 1. Correlation of the percentage of small colonies on MOX with the presence of agglutination titers and plasmids of strain WA

% of small colonies on MOX agar[a]	Agglutination titer[b]	Plasmids[c]
3	60	−
4	<480	−
23	3,840	+
24	7,680	+
29	<480	+
33	<480	−
34	3,840	+
49	61,440	+
55	7,680	+
71	61,440	+
81	61,440	+

[a] Averages from three to six plates at 100 CFU per plate or less.
[b] Titers are expressed as reciprocals of the highest antiserum dilution that gives a positive agglutination result with WA-SAA (8).
[c] Plasmids were isolated by the methods of Birnboim and Doly (2). An 18-h culture (2 ml; equivalent to an optical density at 520 nm of 3) grown in TSB was used for plasmid isolation. The same culture was used for both the agglutination test and enumeration of small colonies on MOX. Electrophoresis procedures were as described by Chang and Doyle (7). The 42-megadalton plasmid was consistently present; however, the 82-megadalton plasmid was not. There was no definitive plasmid profile among the isolates to correlate the 82-megadalton plasmid with virulence characteristics.

attain a diameter of 2 to 2.5 mm after 40 to 48 h), and continue to enlarge, as do the large colonies upon further incubation. Perhaps the small colonies were a transient plasmid-carrying population originating from a plasmid-bearing cell whose progeny were overgrown by plasmidless derivatives in the same colony. The small colonies picked at 24 h after growth on MOX did not contain virulence plasmids and had no detectable agglutination titer (<30).

Several of the small MOX-grown colonies from strain WA were tested further for mouse lethality. Their characteristics are described below. Male BALB/c mice (6 to 8 weeks old) were injected intraperitoneally with 2×10^7 to 5×10^7 CFU of live bacteria (grown overnight in TSB at 37°C) and observed for 3 weeks. Strain WA (from stock culture, not from MOX) killed mice within 5 days, whereas a culture of a large MOX-grown colony of WA or of avirulent strain WA-ETBR did not kill or cause sickness in mice. Five groups of three to four mice each were injected with small MOX-grown colonies (Table 2). Mice that died within the 3-week period were confirmed to have *Y. enterocolitica* in their liver and spleen. All of the surviving mice were autopsied and examined for abnormalities, and the liver, spleen, and mesenteric lymph nodes were cultured for *Y. enterocolitica*. These results indicated that some, but not all, of the small colonies were virulent. Additionally, virulence was greatly reduced compared with that of the parental strain, WA. These results thus suggest that MOX is selective against virulent isolates of this organism.

TABLE 2. Mouse lethality tests of small-colony isolates from MOX of strain WA (WA-S1 through WA-S5) and parental WA and avirulent WA-ETBR

Strain or isolate[a]	Cumulative death in week:			Survival in week 3	
	1	2	3	No. of survivors	Organ culture[b]
WA	4/4			0/4	NA
WA-ETBR	0	0	0	4/4	Negative
WA-S1	0	2/4	3/4	1/4	Negative
WA-S2	0	0	0	4/4	Negative
WA-S3	0	0	2/3	1/3	Positive
WA-S4	1/3	3/3		0/3	NA
WA-S5	0	0	1/3	2/3	Positive

[a] WA-S1 through WA-S5 were small-colony isolates from MOX of strain WA. All strains and isolates were maintained on Trypticase soy agar (BBL Microbiology Systems) slants.
[b] Spleen, liver, and mesenteric lymph nodes were cultured from surviving mice for isolation of *Y. enterocolitica*. NA, Not applicable.

The characteristics of isolates recovered from mice were compared with those of the original small colony cultures used for injection (Table 3). Interestingly, cultures recovered from infected mice both were WA-SAA agglutination positive and had a large proportion of small colonies when plated on MOX. These cultures also contained the virulence plasmid(s). In contrast, the only virulence trait manifested in the original isolates was the presence of small colonies on MOX. However, even in this case, the virulence potential could not be predicted based on the presence, or the percentage, of small colonies on MOX. Isolate WA-S2, for instance, had a similar ratio of small colonies as those of the other four isolates, yet was avirulent in mice. Hence, differences in cumulative deaths and the degree of mouse virulence were not reflected in the proportion of small-colony types present in the inoculum.

Results suggest that passage of cultures through mice greatly enhances the expression of virulence traits, such as plasmid content, WA-SAA agglutination, and the preponder-

TABLE 3. Comparison of cultures used for mouse lethality tests and cultures recovered from infected mice for agglutination titer, plasmid content, and percentage of small colonies on MOX

Strain or isolate[a]	Comparison of cultures					
	Used for mouse lethality tests			Recovered from infected mice[b]		
	Agglutination titer[c]	Virulence plasmids[d]	% Small colonies on MOX	Agglutination titer[c]	Virulence plasmids[d]	% Small colonies on MOX
WA	1,920	+	58	3,840	+	ND
WA-ETBR	<2	−	0	NA	NA	NA
WA-S1	<2	−	14	3,840	+	86
WA-S2	<2	−	13	NA	NA	NA
WA-S3	<2	−	9	1,920	+	83
WA-S4	<2	−	8	3,840	+	94
WA-S5	<2	−	11	1,920	+	90

[a] See Table 2, footnote *a*.
[b] Cultures were obtained from isolates of organs plated on MacConkey agar (Difco Laboratories) or yersinia selective agar (Oxoid Ltd.), and biochemical characteristics were confirmed by the API-20E diagnostic system (Analytab Products). ND, Not determined; NA, not applicable (no organism was isolated).
[c] See Table 1, footnote *b*.
[d] See Table 1, footnote *c*.

ance of small colonies developing on MOX. Results also indicate that virulence potential cannot always be identified by the above in vitro methods. The antigenic nature of the virulence-specific outer membrane polypeptides (7) and their relationship with the virulence of these isolates are currently under investigation. Since virulence is probably a multifactorial phenomenon, it is likely that additional virulence factors remain to be identified.

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