Assay of Crystal Violet Binding for Rapid Identification of Virulent Plasmid-Bearing Clones of *Yersinia enterocolitica*

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A rapid, reliable, and simple method based on the binding of crystal violet (CV) is described for differentiating virulence-plasmid-bearing strains of *Yersinia enterocolitica* from their plasmidless derivatives. As with other plasmid-mediated properties of this organism, the binding of CV occurs at 37°C but not at 25°C. The CV-binding technique provides a simple and efficient means of screening *Y. enterocolitica* for virulence and for identifying individual plasmid-bearing colonies.

Human pathogenic strains of *Yersinia enterocolitica* harbor a particular species of 40- to 48-megadalton plasmid DNA (1, 15, 20, 21). Evidence for the direct involvement of the plasmid in the virulence of the organism was described recently by Heesemann et al. (4). A number of phenotypic characteristics associated with the virulence plasmid have also been described (3, 7–15, 20, 21). Virulent *Yersinia* strains dissociate into virulent and avirulent clones after cultivation. Such dissociation is associated with plasmid loss, an event which is favored by subculturing at 37°C (1, 8, 15, 20, 21). Consequently, plasmid loss results in the loss of virulence and the concomitant disappearance of the associated phenotypic characteristics. Many plasmid-mediated properties have been used to distinguish between virulent and avirulent strains of *Y. enterocolitica*, including colony morphology (12, 13), autoagglutination (10, 19), detachment of cells in culture (11), serum resistance (14), hydrophobicity (8, 9), and virulence characteristics in animals (16, 18). A colony hybridization technique has also been described for the detection of virulent strains of *Y. enterocolitica* (6). However, most of these experimental procedures are costly, time-consuming, complex, and impractical for routine diagnostic use or in field laboratories. The Congo red pigmentation assay provides a simple and efficient means of screening *Y. enterocolitica* for virulence (16). However, a recent study (17) reported that a significant number of avirulent food-borne isolates were Congo red positive. Observations in our laboratory parallel these findings.

The objective of the current study was to develop an alternate method for differentiating virulent *Y. enterocolitica* strains. We report on the correlation of virulence in *Y. enterocolitica* with the ability of pregrown colonies to bind crystal violet (CV) and the development of a simple assay based on this characteristic.

MATERIALS AND METHODS

Bacteria and growth conditions. Plasmid-bearing virulent strains (P^+) of *Y. enterocolitica*, kindly provided by W. E. Hill, Food and Drug Administration, Washington, D.C., and the avirulent plasmidless derivatives (P^-) used in this study are listed in Table 1. The P^- strains were obtained from large, flat colonies which emerged spontaneously from P^+ cultures growing at 37°C on brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) agarose as described by Lachica and Zink (8). The cultures were stored as cell suspensions at −20°C in 50% glycerol and cultivated in BHI broth at 25°C for 18 h unless otherwise indicated.

Virulence and in vitro properties associated with virulence. Swiss Webster albino male mice (15 to 20 g each) were used to determine the virulence of each P^+ and P^- strain. The mice were pretreated with 5 mg of iron-dextran (Imferon; Merrell Dow Research Institute, Cincinnati, Ohio) and 5 mg of desferroxamine (Desferal; CIBA-GEIGY Corp., Suffern, N.Y.), infected orally, and examined for diarrhea (18). Calcium dependence of the bacteria was tested by growth on agar containing magnesium oxalate (MOX agar) (5). Autoagglutination was determined by the method of Laird and Cavanaugh (10) with Eagle minimal essential medium supplemented with 10% fetal bovine serum. Hydrophobicity was examined as described by Lachica and Zink (8). The Congo red acid-morpholinepropanesulfonic acid pigmentation (CRAMP) agar was prepared as previously described by Prpic et al. (16).

Plasmid screening. The presence of plasmid DNA in *Y. enterocolitica* was determined as described by Birnboim and Doly (2).

CV-binding assay. The ability of strains of *Y. enterocolitica* to bind CV was tested as follows. The P^+ and P^- strains were grown separately in BHI broth for 18 h at 25°C with shaking. The cells were diluted to a concentration of 10^3 cells per ml (determined by A_{600}) and surface plated on BHI agar (Difco) by using a model B spiral plater (Spiral System, Cincinnati, Ohio). The plates were incubated at 25 or 37°C for 30 h. They were then gently flooded with 8 ml of a 85-μg/ml solution of CV (Difco) for 2 min and decanted. The binding of CV to P^+ colonies was observed by their dark-violet appearance, while P^- colonies failed to bind the dye and remained white (Fig. 1). After performing the CV-binding assay, we examined the colony morphology of P^+ and P^- strains further by using a low-magnification (×7) stereomicroscope equipped with a light source (model ASZ30L3; Bausch & Lomb, Inc., Rochester, N.Y.). Photographs of colonies were made for permanent records. All the steps described above were performed within 15 min, since

TABLE 1. Virulence and virulence-associated properties of plasmid-bearing strains of *Y. enterocolitica* and their plasmidless derivatives

Strain	Serotype	CV binding		Diarrhea[a] in mice	CAD[b]	AA[c]	Hydrophobicity	Growth on CRAMP agar	Plasmid (40 to 45 megadaltons)
		37°C	25°C						
GER	O:3	+	−	+	+	+	+	+	+
GER-C	O:3	−	−	−	−	−	−	+	−
EWMS	O:3	+	−	+	+	+	+	+	+
EWMS-C	O:3	−	−	−	−	−	−	+	−
PT18-1	O:5, O:27	+	−	+	+	+	+	+	+
PT18-1-C	O:5, O:27	−	−	−	−	−	−	+	−
O:TAC	O:TACOMA	+	−	+	+	+	+	+	+
O:TAC-C	O:TACOMA	−	−	−	−	−	−	+	−
WA	O:8	+	−	+	+	+	+	+	+
WA-C	O:8	−	−	−	−	−	−	+	−

[a] Fecal material consistency was liquid; diarrhea was observed on days 4, 5, 6, and 7, followed by death day 8 postinfection for serogroup O:8; for three other serogroups (O:3, O:5, O:27, and O:TACOMA), diarrhea was observed on days, 5, 6, and 7 postinfection, and death did not occur.
[b] CAD, Calcium dependence.
[c] AA, Autoagglutination.

pigmentation differentiation between the P^+ and P^- colonies diminishes with time.

RESULTS

CV-binding test. The P^+ and P^- GER strains (serotype O:3) were used for standardization of optimal conditions for the CV-binding technique. The P^+ strain bound CV, producing dark-violet colonies (CV^+) after the cells had grown for 30 h at 37°C (Fig. 1A). The P^- colonies did not bind CV and remained white (CV^-) (Fig. 1B). Colony morphologies of the CV^+ and CV^- strains were examined further by using a low-magnification stereomicroscope. The CV^+ strain formed small, convex, shiny, dark, opaque colonies, whereas the CV^- strain formed shiny, translucent, flat, white colonies. When cells were grown at 25°C under similar conditions, neither P^+ nor P^- strains bound CV (Table 1). The optimum concentration of CV in the binding assay was found to be 85 μg/ml. At reduced concentrations, the color intensity of the P^+ colonies was low. At elevated concentrations, the background color intensity was high. CV binding was also tested with mixed cultures of the P^+ and P^- strains to assess the ability of the technique to quantitatively differentiate P^+ and P^- cells (Table 2). The results demonstrate that the CV binding could be used to identify individual plasmid-bearing colonies from mixed culture on a BHI agar plate. The efficiency of this assay was about 94.4% as calculated from the number of added P^+ cells. The utility of the CV-binding technique for distinguishing virulent strains of *Y. enterocolitica* was assessed further by examining five P^+ strains and their P^- derivatives, representing four serotypes, under the conditions described above. All P^+ strains of different serotypes grown at 37°C responded positively to the CV-binding screening test, with typical dark-violet colony morphology, and the corresponding isogenic P^- strains did not bind to CV and remained whitish (Table 1).

Correlation between CV binding, virulence, and virulence-

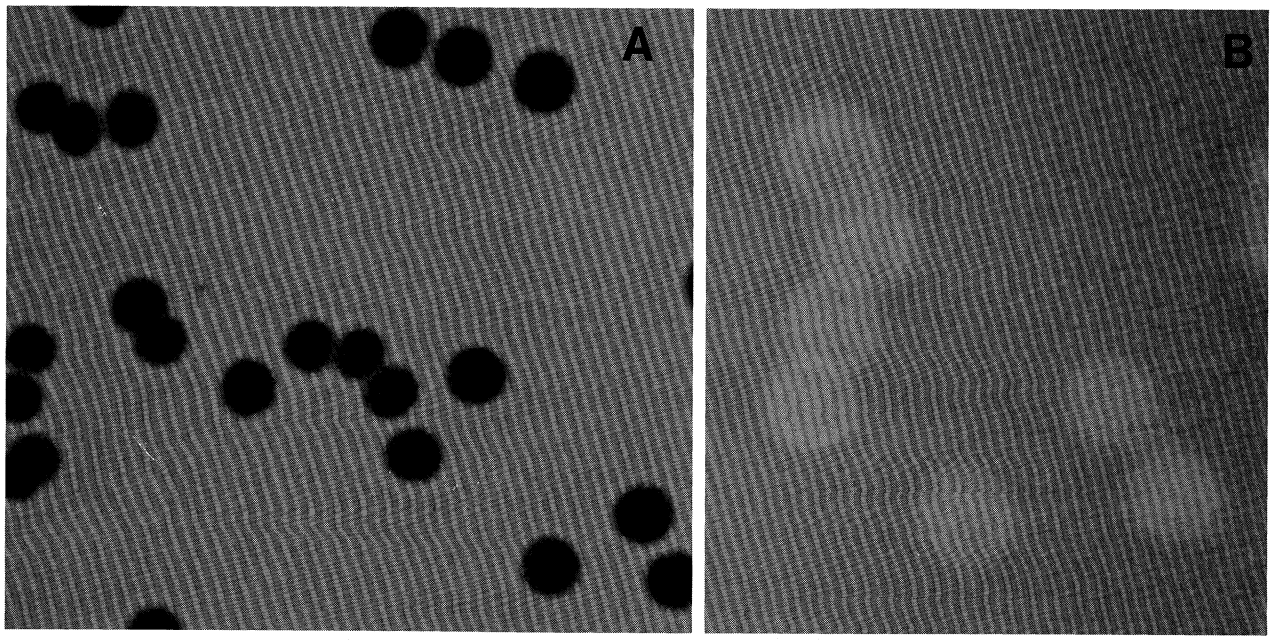

FIG. 1. CV binding to colonies of *Y. enterocolitica* GER (serotype O:3) when cells were grown on BHI agar for 30 h at 37°C. (A) Virulent P^+ cells, showing dark-violet colonies. (B) Avirulent P^- cells, showing white colonies. The concentration of CV used in the binding assay was 85 μg/ml.

TABLE 2. Efficiency of CV binding in mixed cultures of virulent and avirulent strains[a]

Sample	Estimated no. of colonies		No. (%) of colonies which bound CV[b]
	Avirulent	Virulent	
A	172		0
B	141	16	16 (100)
C	131	31	29 (93)
D	85	56	56 (100)
E	72	98	92 (93)
F	53	124	103 (83)
G	22	130	124 (94)
H		175	173 (98)

[a] Virulent cells of *Y. enterocolitica* GER (serotype O:3) were mixed in various ratios with cells from the plasmidless GER strain and surface plated on BHI agar. The mixed colonies were incubated at 37°C for 30 h. The number of virulent colonies was determined by the CV-binding technique at a concentration of 85 μg of CV per ml.

[b] Average percent binding was 94%.

associated characteristics. Virulent and avirulent (derived from each P^+ strain) strains from four serotypes were examined for virulence and various virulence-associated characteristics. The CV binding was correlated with virulence as shown by oral infection causing diarrhea in iron-overloaded mice (Table 1). Although lethality resulting from the oral infection has been used as a test for virulence of *Y. enterocolitica* (18), we found that only the CV^+ WA strain of serogroup O:8 evoked clear-cut lethality to mice as well as diarrhea. All of the CV^+ strains described in this study were also examined for a number of virulence-associated properties, including calcium dependence, autoagglutination, hydrophobicity, and Congo red pigmentation. In every case except Congo red pigmentation, CV^+ but not CV^- strains displayed appropriate virulence-associated characteristics (Table 1). Both P^+ isolates and their P^- derivatives gave red colonies on a medium containing Congo red, as described by Prpic et al. (16) (Table 1).

Correlation between CV binding and plasmid carriage. All five virulent strains from four serotypes harbored plasmids in the size range of 40 to 45 megadaltons (data not shown). Plasmids were not detected in P^- derivatives. Furthermore, in all strains examined, loss of plasmid resulted in loss of CV binding, as well as of other virulence properties.

DISCUSSION

A wide range of experimental procedures has been applied to the determination of virulence among the strains of *Y. enterocolitica*. Although the Congo red pigmentation method is simple, it cannot always differentiate virulent (P^+) and avirulent (P^-) strains (17). DNA colony hybridization (6) has been successfully applied to the detection of virulent strains, with plasmid DNA gene fragments as the genetic probe. However, this technique is complex and time-consuming. Moreover, the use of a ^{32}P-labeled DNA probe necessitates the frequent preparation of the probe, which is inconvenient and expensive, as well as requiring the handling of millicurie levels of radioactive material, which is impractical for many field laboratories. The use of less hazardous ^{3}H-labeled, ^{35}S-labeled, and nonradioactive DNA probes provides lower sensitivity for detection purposes. Although the virulence of certain serogroups can be demonstrated effectively by using laboratory animals (1, 16–18), these tests are not suitable for routine diagnostic use. Other in vitro methods such as serum resistance (14), autoagglutination (10, 15), and the tissue culture detachment assay (11) are laborious, and the widely used calcium dependence test has the disadvantage that virulent strains grow poorly, forming pinpoint colonies on MOX agar (3, 16, 17). Our results indicate that binding of CV to virulent strains allows the rapid and simple differentiation of virulent and avirulent strains of *Y. enterocolitica*. This assay takes about 3 to 5 min, independent of the time for growth of the culture, and can effectively demonstrate the presence of virulent cells in cultures containing predominantly P^- cells. Such cultures are not uncommon in clinical laboratories in which incubation at 37°C is standard procedure. Microscopic observation is not necessary to distinguish between virulent and avirulent strains. Since CV diffuses throughout the agar plate and all the colonies within a relatively short time, it is recommended that users obtain photographs to serve as permanent records. Thus, the present technique offers distinct advantages, since it does not require special equipment and can be used effectively with large numbers of cultures.

CV binds to the colonies of the virulent strains only when cells are grown at 37°C and is similar to other virulence-associated properties, such as occurrence of outer membrane proteins (15), fibrillae (7, 15), autoagglutination (10, 19), serum resistance (14), surface charge, hydrophobicity (8, 9), and calcium dependence (3), all of which occur at 37°C. It is surmised that the cell surface components facilitate the binding of CV to virulent strains, although the nature of such cell surface components has not been fully elucidated.

The ability of colonies to bind CV appears to be encoded by the virulence-associated plasmid of *Y. enterocolitica*. All the CV^+ strains examined harbored a plasmid and demonstrated other virulence-associated attributes that have been reported to be plasmid determined. In contrast, none of the CV^- strains contained any plasmid or showed virulence. Although virulence was plasmid mediated in all strains examined, the plasmids involved differed in molecular weight. Thus in epidemiological studies, it is not sufficient to search for plasmids of a particular molecular weight as an indicator of *Y. enterocolitica* virulence. The correlation of virulence with the ability to bind CV was most clearly demonstrated by the finding that only CV^+ strains, regardless of serogroup, were virulent to mice.

In conclusion, the CV-binding technique permits the rapid identification of the *Y. enterocolitica* bacterial colonies containing plasmid-bearing cells. Further studies are required to determine the extent to which CV binding is shared by avirulent strains of *Y. enterocolitica* and other environmental *Yersinia* spp. (21).

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