

**SIMPLE AND RAPID METHODS FOR IDENTIFICATION OF
VIRULENT PLASMID-BEARING CLONES OF
*YERSINIA ENTEROCOLITICA*¹**

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ABSTRACT

*The detection of cold-tolerant plasmid-bearing human pathogenic strains of *Yersinia enterocolitica* depends on the expression of various plasmid-associated characteristics. However, techniques based on these characteristics have limited reliability and are impractical for diagnostic use. Two rapid, reliable, and simple methods based on the binding of crystal violet and Congo red for identification and differentiation of *Y. enterocolitica* containing virulent plasmid-bearing strains are discussed. These dye binding assays can be used to assess the pathogenicity of *Y. enterocolitica* isolates from a variety of sources for identification of individual plasmid-bearing clones.*

INTRODUCTION

The association of human illness with consumption of food contaminated with *Yersinia enterocolitica* is well-documented (Doyle and Cliver 1990; Lee *et al.* 1990; Kapperud 1991). Since yersiniae grow at low temperatures, refrigerated foods are potential vehicles for the growth of the organisms (Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985). Strains of all serotypes implicated in human disease harbor a plasmid of 40–45 megadaltons that contributes

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²To whom correspondence should be addressed. Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

to virulence of the bacterium and is referred to as the virulence plasmid (Brubaker 1991; Cornelis *et al.* 1987; Kapperud 1991; Miliotis *et al.* 1989; Portnoy and Martinez 1985). A number of temperature dependent phenotypic characteristics including mouse virulence have been associated with the virulence plasmid and have been used to differentiate between virulent and avirulent strains of *Y. enterocolitica* (Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985; Kwaga and Iversen 1991; Robins-Browne 1989; Farmer *et al.* 1992). However, the physiological traits associated with the virulence plasmid are expressed only at 37C, which also fosters the loss of the virulence plasmid and the concomitant disappearance of the associated phenotypic virulence characteristics (Brubaker 1991; Cornelis *et al.* 1987; Doyle and Cliver 1990; Kapperud 1991; Miliotis *et al.* 1989; Portnoy and Martinez 1985). The plasmid is stable in cells maintained at 25–28C. Because of the instability of the virulence plasmid at 37C, it is difficult to isolate plasmid-bearing virulent strains after initial detection. As a consequence, detection has been hampered in clinical, regulatory and quality control laboratories that employ an incubation temperature of 37C for isolation/detection of the organism. For example, such difficulties were reported by the California health department and U.S. Food and Drug Administration (FDA) in recent cases of yersiniosis in Los Angeles County, CA (Weagant 1992). The instability of the plasmid can lead to confusion concerning whether one is dealing with virulent or nonvirulent strains. The mouse virulence assay (Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985) could be used for the isolation of plasmid-bearing virulent strains of *Y. enterocolitica*, but the procedure is expensive and inconvenient for routine use. There is no simple, reliable and rapid method available for both identification and isolation based on plasmid-associated properties described in the literature (Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985; Kwaga and Iversen 1991; Robins-Browne *et al.* 1989; Farmer *et al.* 1992). The purpose of this paper is to review the research on the development of alternative methods for identification of plasmid-bearing virulent strains of *Y. enterocolitica* based on the ability of virulent strains of *Y. enterocolitica* to bind crystal violet (CV) and Congo red (CR).

Detection of Plasmid-Bearing Virulent Strains of *Y. enterocolitica*

A wide variety of experimental procedures including *in vitro* methods such as colony morphology, agglutination, serum resistance, tissue culture detachment, hydrophobicity, and calcium dependency, [also known as low-calcium response (Lcr)] has been applied to the determination of virulence in strains of *Y. enterocolitica* (Doyle and Cliver 1990; Farmer *et al.* 1992; Kapperud 1991; Kwaga and Iversen 1991; Portnoy and Martinez 1985; Robins-Browne *et al.* 1989). These methods require specific reagents and conditions and do not give clearcut results.

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However, most of these procedures are costly, time-consuming, complex, and impractical for routine diagnostic use, particularly in field laboratories. Although the virulence of certain serogroups can be demonstrated effectively using laboratory animals (Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985), this test is not suitable for routine diagnostic use. DNA colony hybridization has also been successfully applied to the detection of virulent strains, with plasmid DNA gene fragments as genetic probes (Miliotis *et al.* 1989; Robin-Browne *et al.* 1989; Kwaga *et al.* 1992). However, this technique is complex and time consuming. A major disadvantage of colony hybridization is the necessity of using a ^{32}P labeled DNA probe. The short life of ^{32}P (14 days) necessitates the frequent preparation of the probe, which is inconvenient and expensive. The method also requires handling of millicurie levels of radioactive material, thus making it less acceptable because of radiation hazards and safety regulations. Use of less hazardous ^3H -labeled or ^{35}S -labeled or nonradioactive DNA probe has lower sensitivity. DNA restriction fragment length polymorphisms (Blumberg *et al.* 1991) and polymerase chain reaction technologies (Kwaga *et al.* 1992) have been used, but they require highly sophisticated and complex techniques. These methods detect only the presence of a specific gene but not the actual presence of the organism. Although virulence is 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.

Development of Dye Binding Techniques

The ability of *Yersinia* to absorb hemin from agar media is correlated with the virulence plasmid (Portnoy and Martinez 1985). This fact led Bhaduri *et al.* (1987, 1991) to postulate that dye binding may be an indication of the presence of the plasmid-bearing virulent cells (P^+). Several dyes including CV were included in the brain heart infusion agar (BHA, Difco Laboratories, Detroit, MI) for the detection of P^+ cells but all of the dyes bound to both virulent and avirulent strains after incubation at 37C for 24 h. Instead, flooding of pregrown colonies of *Y. enterocolitica* at 37C with CV solution at a concentration of 100 μg per ml (Bhaduri *et al.* 1987; Bhaduri 1990) indicated that P^+ cells bound CV and produced dark-violet colonies (Fig. 1A, Table 1). Plasmidless avirulent (P^-) colonies did not bind CV and remained white (Fig. 1B, Table 1). The CV flooding and binding assay takes about 3–5 min. Since CV ultimately diffuses throughout the agar plate and all the colonies turn purple within a relatively short time, it is recommended that users take photographs to serve as permanent records during early phase of flooding.

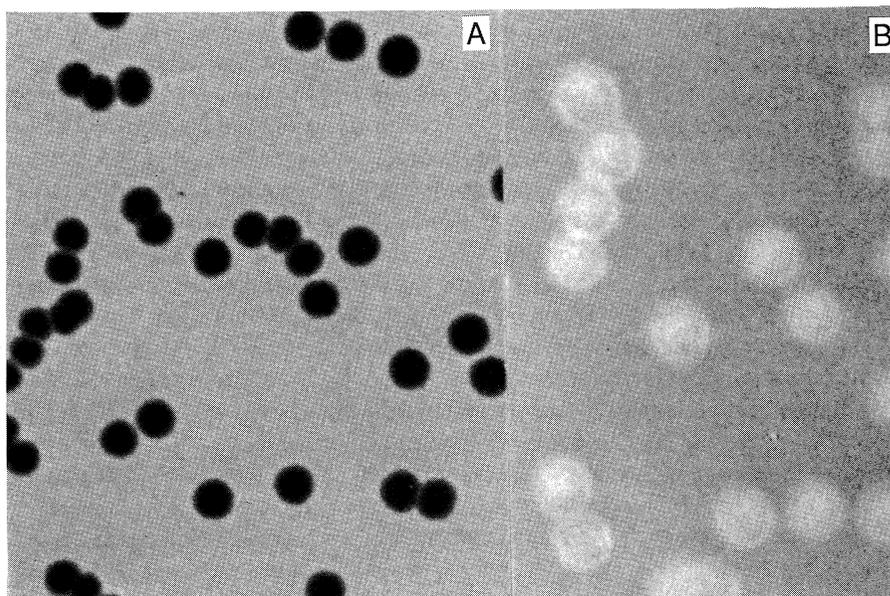


FIG. 1. CV BINDING OF COLONIES OF *Y. ENTEROCOLITICA* GER (SEROTYPE O:3)
GROWN ON BHA FOR 24 H 37C

After incubation plates were flooded with a 100 μ g per ml CV solution.

(A) Virulent P⁺ cells showing small dark-violet colonies.

(B) Avirulent P⁻ cells showing large white colonies. (Bhaduri *et al.* 1987)

After the CV binding technique was developed, it was found that there were disadvantages to this technique: (1) it required an extra step of flooding; (2) the CV solution kills the cells in the colonies. So an alternative approach was taken. CR had been used unsuccessfully to screen *Y. enterocolitica* for virulence (Prpic *et al.* 1985; Bhaduri *et al.* 1987), so more specific conditions for the binding of CR to P⁺ cells were evaluated. Because agarose is a purer form of agar, it has been found that its calcium level is lower than agar (Bhaduri *et al.* 1990). Both agar and agarose were used as gelling agents in brain heart infusion (BHI Difco) to attain low and high levels of calcium in the media (Bhaduri *et al.* 1990). Taking advantage of the noninhibitory nature of CR on the growth of bacteria, the dye was added at a concentration 75 μ g per ml prior to autoclaving of BHI containing either agarose or agar. The addition of CR in the media did not change the concentration of calcium in the respective media (Table 2). The CR containing low and high calcium media were used for the determination of CR uptake by P⁺ strains (Bhaduri *et al.* 1991). When P⁺ and P⁻ strains were cultivated at 37C for 12–24 h on these two media, only CR containing low-calcium BHI

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TABLE 1.
CORRELATION BETWEEN DYE BINDING TECHNIQUES, VIRULENCE AND
VIRULENCE-ASSOCIATED PROPERTIES OF ORIGINAL AND RECOVERED*
PLASMID-BEARING STRAINS OF *Y. ENTEROCOLITICA*

Strain	Serotype	CV	CR	Lcr	AA ^a	HP ^b	Plasmid (40-45 megadaltons)	Diarrhea ^c in mice
		Binding	Binding					
GER	0:3	+	+	+	+	+	+	+
GER-RE	0:3	+	+	+	+	+	+	+
GER-C	0:3	-	-	-	-	-	-	-
EWMS	0:13	+	+	+	+	+	+	+
EWMS-RE	0:13	+	+	+	+	+	+	+
EWMS-C	0:13	-	-	-	-	-	-	-
PT18-1	0:5,0:27	+	+	+	+	+	+	+
PT18-1-RE	0:5,0:27	+	+	+	+	+	+	+
PT18-1-C	0:5,0:27	-	-	-	-	-	-	-
O:TAC	0:TACOMA	+	+	+	+	+	+	+
O:TAC-RE	0:TACOMA	+	+	+	+	+	+	+
O:TAC-C	0:TACOMA	-	-	-	-	-	-	-
WA	0:8	+	+	+	+	+	+	+
WA-RE	0:8	+	+	+	+	+	+	+
WA-C	0:8	-	-	-	-	-	-	-

*Recovered strains are designated as RE.

^aAA: Autoagglutination.

^bHP: Hydrophobicity.

^cFecal material consistency was liquid; diarrhea was observed on days 3, 4, and 5 post infection.

Methods used in the assays are as described previously (Bhaduri *et al.* 1987; Bhaduri *et al.* 1991).

agarose medium (CR-BHO) demonstrated two types of readily discernible colonies. The P⁺ cells absorbed CR and formed red pinpoint colonies (CR⁺) (Fig. 2A, Table 1). The P⁻ cells failed to bind the dye and formed much larger white or light orange colonies (CR⁻) (Fig. 2B, Table 1). The size and colony morphologies of P⁺ strain in CR-BHO also showed Lcr (Bhaduri *et al.* 1990, 1991;

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TABLE 2.
ESTIMATION OF CALCIUM IN THE MEDIA TO DEFINE LOW-CALCIUM AND
HIGH-CALCIUM MEDIA

Medium	Calcium Concentration in μM^*	Medium
BHO	238 (Low)	CR-BHO
BHA	1500 (High)	CR-BHA
BHI Broth	245 (Low)	BHI Broth

*The concentration of calcium in each media was determined by atomic absorption analysis (Bhaduri *et al.* 1990).

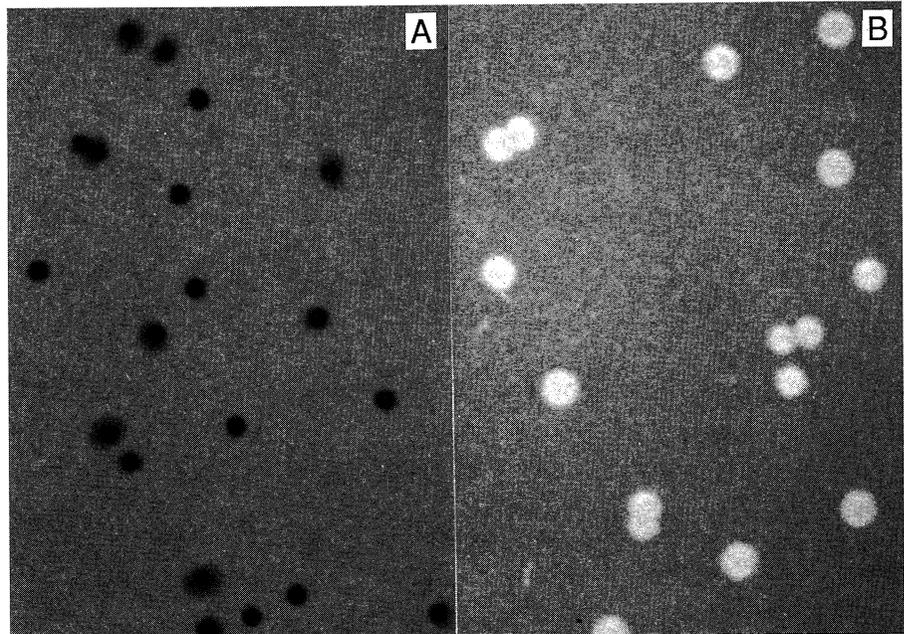


FIG. 2. CR BINDING OF COLONIES OF *Y. ENTEROCOLITICA* GER (SEROTYPE O:3) CELLS
GROWN ON CR-BHO FOR 24 H AT 37C

(A) Virulent P^+ cells showing pinpoint red colonies. (B) Avirulent P^- cells showing large white or light orange colonies. The concentration of CR used in the binding assay was $75 \mu\text{g/ml}$. (Bhaduri *et al.* 1991)

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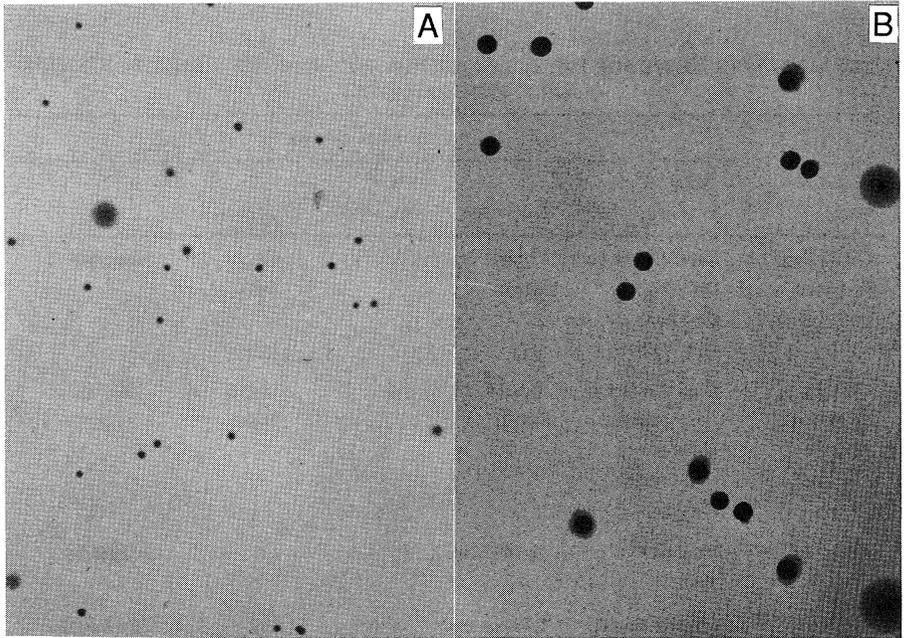


FIG. 3. CR BINDING OF VIRULENT CELLS OF *Y. ENTEROCOLITICA* GER (SEROTYPE O:3) GROWN ON CR-BHO FOR 24 AND 48 H AT 37C

(A) White border around the red center of the colony after 24 h of incubation. (B) White wide border around the red center of the colony after 48 h of incubation. (Bhaduri *et al.* 1991)

Brubaker 1991). Another characteristic feature of the CR binding technique for P^+ strains is the appearance of a white opaque circumference around the red center after ≥ 24 h of incubation (Fig. 3A and 3B). It is important to note that cells in the red center contain the virulence plasmid, whereas, cells in the white surrounding border do not contain the plasmid since they have lost CR binding property. This observation was also confirmed by hybridization with specific DNA probe (Bhaduri *et al.* 1991). It means that initially cells do not lose the virulence during growth at 37C but do so on prolonged incubation. This colonial characteristic is another parameter that can be used for the identification of virulent strains of *Y. enterocolitica*.

Identical results were obtained with other *Y. enterocolitica* serotypes (Table 1). The binding of CV and CR to virulent strains consistently allowed the ready differentiation of virulent and avirulent strains of *Y. enterocolitica*. As with other plasmid-mediated properties of this organism, the binding of the dyes occurs at 37C but not at 25C. A positive response in the CV and CR binding tests was

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TABLE 3.
COMPARISON OF TESTS FOR DETECTION OF PLASMID-BEARING VIRULENT STRAINS
OF *Y. ENTEROCOLITICA*

Detection Method	Comparison Criteria				
	Detection Principle	Specificity	Recovery	Convenience	Cost
CV Binding Technique	Uses Virulences Determinants Based on Dye Binding	Virulent Strains	No	Simple and Rapid	Inexpensive
CR Binding Technique	Uses Virulences Determinants Based on Dye Binding and Lcr	Virulent Strains	Yes	Simple and Rapid	Inexpensive
API	Biochemical Tests	<i>Yersinia</i> spp.	No	No	Expensive
CIN agar	Medium Based	<i>Yersinia</i> spp.	No	No	Expensive
SYS	Enzyme Based	No	No	No	Expensive
Vitek	Medium Based	No	No	No	Expensive
Progen	Uses Antibody	<i>Yersinia</i> spp.	No	No	Expensive

API: Analytical Products, Plainview, NY.

SYS: Analytical Products, Plainview, NY.

Vitek: Hazelwood, MO.

Progen: Heidelberg, Germany.

correlated with the presence of plasmid as well as with a number of virulence associated properties including mouse virulence (Table 1). The present techniques do not require special equipment and can be used to screen large numbers of cultures. These tests can assist both small and large laboratories in the identification of clusters of cases of yersiniosis. The techniques have also made it possible to study the effects of food processing conditions on the stability of the virulence plasmid; including temperature (Bhaduri *et al.* 1988), salt and pH level (Bhaduri and Mertz 1989), and atmosphere (Bhaduri and Turner-Jones 1993). Thus, CV and CR binding assays offer distinct advantages over currently available com-

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TABLE 4.
EFFICIENCY OF CV BINDING IN MIXED CULTURES OF VIRULENT AND
AVIRULENT STRAINS^a

Sample	Estimated No. of Colonies in the Mixture		No (%) of Colonies Bound to CV ^b
	Avirulent	Virulent	
A	172	0	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	0	175	173 (98)

^aVirulent cells of *Y. enterocolitica* GER (serotype 0:3) were mixed in various ratios with cells from the plasmidless GER strain and surface plated on BHI agar. The mixed colonies were incubated at 37C for 24 h. The number of virulent colonies was determined by the CV-binding technique at a concentration of 100 µg CV per ml.

^bAverage percent binding was 94.

mercial tests (Table 3). These two assays can effectively detect the presence of virulent P⁺ cells in cultures containing predominantly P⁻ cells (Tables 4 and 5). Such cultures are not uncommon in clinical laboratories where incubation at 37C is standard procedure (Weagant 1992). This was demonstrated by the FDA investigation to assess a recent *Yersinia* outbreak in Los Angeles County, CA. The CR binding technique successfully detected the plasmid-bearing virulent cells, which made up only 0.3% of the total population of yersinae in the clinical samples (Weagant 1992). Thus, this technique is highly sensitive and can be used to detect very low levels of P⁺ cells in a mixture of P⁻ cells.

An additional value of the CR binding technique is that it can be used to isolate viable P⁺ cells since the use of CV leads to the death of the cells. This permits the recovery of P⁺ cells even in cultures that have grown at 37C. The recovery technique has been applied successfully to five serotypes of *Y. enterocolitica* and varied from 5 to 95% (Table 6) indicating strain variation in the stability of the

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TABLE 5.
EFFICIENCY OF CR BINDING IN MIXED CULTURES OF VIRULENT AND
AVIRULENT STRAINS^a

Sample	Estimated No. of Colonies in the Mixture		No (%) of Colonies Bound to CR ^b
	Avirulent	Virulent	
CR-BHO:A	67	4	4 (100)
B	63	10	10 (100)
C	49	30	30 (100)
D	35	50	50 (100)
E	21	70	70 (100)
F	7	90	90 (100)

^aVirulent cells of *Y. enterocolitica* GER (serotype O:3) were mixed in various ratios with cells from plasmidless GER strain and surfaced plated on CR-BHO. The mixed colonies were incubated at 37C for 24 h. The number of virulent colonies were determined by the appearance of red pinpoint colony.

^bAverage percent of efficiency was 100.

TABLE 6.
RECOVERY OF PLASMID-BEARING VIRULENT STRAINS AFTER DETECTION
BY CR BINDING TEST

<u>Strain</u>	<u>Serotype</u>	<u>Percentage Recovery</u>
GER	O:3	90-95
EWMS	O:3	3-6
PT18-1	O:5,O:27	90-95
O:TAC	O:TACOMA	3-5
WA	O:8	50-60

P⁺ strains were recovered as described by Bhaduri *et al.* (1991). The initial detection and percent recovery of plasmid-bearing cells was determined by CV and CR binding techniques.

plasmid. The recovered P⁺ strains show all the plasmid-associated properties including the virulence in mouse (Table 1). By using this recovery technique FDA investigators were able to recover and enhance the level of plasmid carriage from

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0.3% to over 92% from the clinical samples obtained from the Los Angeles County, CA, outbreak of *Y. enterocolitica* (Weagant 1992). Thus the recovery technique is useful to isolate and enrich viable P⁺ cells even if they are present at very low levels in a mixture of cells. Therefore, it allows further confirmation on the presence of P⁺ cells and subsequent investigation.

CONCLUSION

The CV and CR binding technique permit rapid and accurate identification of *Y. enterocolitica* bacterial colonies harboring the virulence plasmid. The detection methods are based on four virulence determinants: (1) CV positive small colony; (2) appearance as pinpoint colony (Lcr); (3) Red colony by CR binding; and (4) the appearance of a white border around the red center of the colony on continued incubation at 37C. The combined use of CV binding and CR-BHO techniques provides a method for accurately differentiating between pathogenic and nonpathogenic *Y. enterocolitica*. In addition, CR binding technique allows rapid isolation of viable plasmid-bearing virulent strains for subsequent investigation.

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