

**USE OF MULTIPLE PLASMID-ASSOCIATED VIRULENCE
DETERMINANTS FOR IDENTIFICATION OF PATHOGENIC
YERSINIA ENTEROCOLITICA¹**

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ABSTRACT

The detection of cold-tolerant plasmid-bearing pathogenic strains of Yersinia enterocolitica depends on the expression of various plasmid-associated phenotypic properties. In this study, low calcium Congo red agarose medium was used as a single multiple test medium to identify pathogenic serotypes of Y. enterocolitica or to indicate the pathogenic potential of individual strains. The assay is based on Congo red uptake, crystal violet binding, low calcium response and hydrophobicity. In general, the efficiency and reliability of this medium was found to be better than existing dye binding techniques for detecting virulent Y. enterocolitica strains.

INTRODUCTION

Yersinia enterocolitica is now recognized as a significant human enteric pathogen (FDA 1992; Brubaker 1991; Cornelis *et al.* 1987; Kapperud 1991). There is considerable confusion in the literature because not all *Y. enterocolitica* strains can cause intestinal infections. Unlike intrinsic pathogens, such as *Salmonella*, strain-to-strain variation has been observed in the pathogenicity of *Y. enterocolitica* (FDA

¹Mention 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.

1992; Brubaker 1991; Cornelis *et al.* 1987; Doyle and Cliver 1990; Kapperud 1991). The pathogenicity of *Y. enterocolitica* is correlated with the presence of a 40–45 megadalton plasmid, which is directly involved with the virulence of the organism (FDA 1992; Brubaker 1991; Cornelis *et al.* 1987; Doyle and Cliver 1990; Kapperud 1991; Portnoy and Martinez 1985). A number of temperature dependent phenotypic characteristics, including mouse virulence, have been associated with the virulence plasmid and, as such, have been used as detection methods to differentiate virulent plasmid-bearing (P^+) and avirulent plasmidless (P^-) *Y. enterocolitica* strains (FDA 1992; Bhaduri 1993; Bhaduri *et al.* 1987; Bhaduri *et al.* 1990, 1991; Farmer *et al.* 1992; Kapperud 1991; Koepfel *et al.* 1993; Kwaga and Iverson 1991; Lachica and Zink 1984; Portnoy and Martinez 1985; Robins-Browne *et al.* 1989).

We developed a simple assay based on crystal violet (CV) binding by pregrown colonies on brain heart infusion (BHI) agar (BHA) (Bhaduri 1993; Bhaduri *et al.* 1987) and low calcium response (Lcr) using low calcium BHI agarose (BHO) (Bhaduri *et al.* 1990). These two techniques require different media to demonstrate these plasmid-associated properties (Bhaduri 1993; Bhaduri *et al.* 1987, 1990). Recently, we described a Congo red (CR) uptake technique in which CR binds to colonies during their growth on BHO containing CR (CR-BHO) (Bhaduri 1993; Bhaduri *et al.* 1991). This medium determines two properties that are plasmid dependent: red colonies by CR uptake and Lcr by the appearance of pinpoint colonies (Bhaduri 1993; Bhaduri *et al.* 1990, 1991). The dye binding techniques have been used to detect P^+ strains and as an indicator of virulence by several investigators (Koepfel *et al.* 1993; Kwaga and Iversen 1991; Robins-Browne *et al.* 1989) and is published in the 7th edition of the Food and Drug Administration's (FDA) *Bacteriological Analytical Manual* (BAM), a compendium of official microbiological methods (FDA 1992). During a recent *Yersinia* outbreak in Los Angeles County, California, the CR binding method was successfully employed by FDA to detect very low numbers of plasmid-bearing virulent cells in clinical samples (one P^+ colony among 300 P^- colonies) (Weagant 1992). The tests have also made it possible to study the effects of food processing conditions on the stability of the virulence plasmid, including temperatures, salt and pH levels, and atmospheres (Bhaduri 1993; Bhaduri and Turner-Jones 1993; Bhaduri *et al.* 1988). Although these simple, rapid techniques detect pathogenic potential in *Y. enterocolitica* (Bhaduri 1993; Bhaduri *et al.* 1987, 1990, 1991), a detection method to determine several traits in a single assay would be more desirable. The present study describes the use of CR-BHO as a single test medium for the simultaneous determination of CR uptake, Lcr, CV binding and hydrophobicity (HP). In addition, the medium allows subsequent isolation of viable P^+ cells.

MATERIALS AND METHODS

Bacteria

Five different P⁺ and their chromosomally isogenic P⁻ strains, GER (Serotype O:3), EWMS (Serotype O:13), PT18-1 (Serotype O:5, O:27), O:TAC (Serotype O:TACOMA) and WA (Serotype O:8), representing five serotypes of *Y. enterocolitica* were used. Detailed description of the strains and sources are given elsewhere (Bhaduri *et al.* 1987).

Preparation of Media

Brain heart infusion (BHI: Difco Laboratories, Detroit, MI) broth and BHA (Difco) were prepared as recommended by the supplier. Since the concentration of calcium is high in BHA (1,500 μ M), this was designated and used as calcium-adequate medium (Bhaduri *et al.* 1990). Calcium-deficient BHO was prepared by adding agarose type V (Sigma Chemical Co., St. Louis, MO) as gelling agent to a final concentration 1.2% to BHI broth supplemented with 0.1% magnesium chloride (Bhaduri *et al.* 1990). Taking advantage of the noninhibitory nature of CR (Sigma), the dye was added at a concentration of 75 μ g/ml to BHO to prepare CR-BHO (Bhaduri *et al.* 1990, 1991).

Virulence and *In Vitro* Properties Associated with Virulence

Mouse virulence test was performed with Swiss Webster albino male mice (15–20 g each). The mice were pretreated with 5 mg of iron-dextran (Imferon: Merrell Dow Research Institute, Cincinnati, OH) and 5 mg of desferroxamine (Desferal; CIBA-Geigy Corp., Suffern, NY), infected orally, and examined for diarrhea (Bhaduri *et al.* 1987). Lcr of the bacteria was tested on BHO (Bhaduri *et al.* 1990). Autoagglutination was determined as previously described (Bhaduri *et al.* 1987) with Eagle minimal essential medium supplemented with 10% fetal bovine serum. HP was examined by latex particle agglutination test (Lachica and Zink 1984).

Plasmid Screening

The presence of plasmid DNA in bacteria was determined by the method of Bhaduri *et al.* (1987).

Dye Binding Assays

After the CV binding and HP by LPA tests were found to be positive in low calcium BHO, it was postulated that CR uptake, Lcr, CV binding and HP could be performed in one single test medium. Low calcium CR-BHO was used as a single test medium to assay these four plasmid-associated virulence determinants. P⁺ and P⁻ cells were grown separately in BHI broth for 18 h at 28C with shaking. Cells were diluted to 10³ cells/ml and surface plated on CR-BHO. After 24 h at 37C, CR binding of P⁺ cells was assessed as red pinpoint colonies with Lcr characteristics (Bhaduri 1993; Bhaduri *et al.* 1990, 1991). CV binding was demonstrated by gently flooding the CR-BHO plate with 8 ml of a 100 µg/ml solution of CV (Difco Laboratories, Detroit, MI) for 2 min (Bhaduri 1993; Bhaduri *et al.* 1987) decanting and observing for pinpoint reddish dark-violet colonies with Lcr characteristics (Bhaduri 1993; Bhaduri *et al.* 1987, 1990, 1991). Calcium-adequate medium, BHA, was used as a positive control for CV binding (Bhaduri 1993; Bhaduri *et al.* 1987).

RESULTS AND DISCUSSION

Virulent P⁺ cells of *Y. enterocolitica* GER (serotype O:3) produced pinpoint reddish dark-violet colonies (Fig. 1A). Avirulent P⁻ cells of *Y. enterocolitica* GER (serotype O:3) produced larger white colonies that remained light colored after exposure to CV (Fig. 1B). This technique has been successfully applied to different serotypes of *Y. enterocolitica*. As positive control for CV binding, P⁺ cells were also surface plated on BHA and BHO and incubated at 37C for 24 h. CV binding was performed as above. Viable P⁺ cells were recovered by removing red pinpoint colonies from 24 h CR-BHO plates prior to CV binding, since the CV solution kills the cells (Bhaduri 1993; Bhaduri *et al.* 1991). The individual pigmented (CV⁺ and CR⁺) colonies from CR-BHO were also tested positive for HP by LPA. The double dye binding of the colonies did not inhibit the LPA test for detection of HP. The binding of CV to the colonies was more intense when cells were grown on CR-BHO than on BHA. The surface components that bind CV were not releasable, since washing of the colonies with water, PBS or 25 µM EDTA did not interfere with the assay. CV binding was still positive when cells were grown up to 48–52 h on CR-BHO, whereas it was negative after 24–30 h on BHA (Bhaduri 1993; Bhaduri *et al.* 1987). This observation is very important when tests are done on injured cells which take a longer time to repair and grow (data not shown). As described previously (Bhaduri 1993, Bhaduri *et al.* 1991), the white border around the red center of colonies that appeared with continued incubation at 37C did not bind to CV and remained white, confirming that cells comprising the white border were P⁻ cells. The cells from the red center

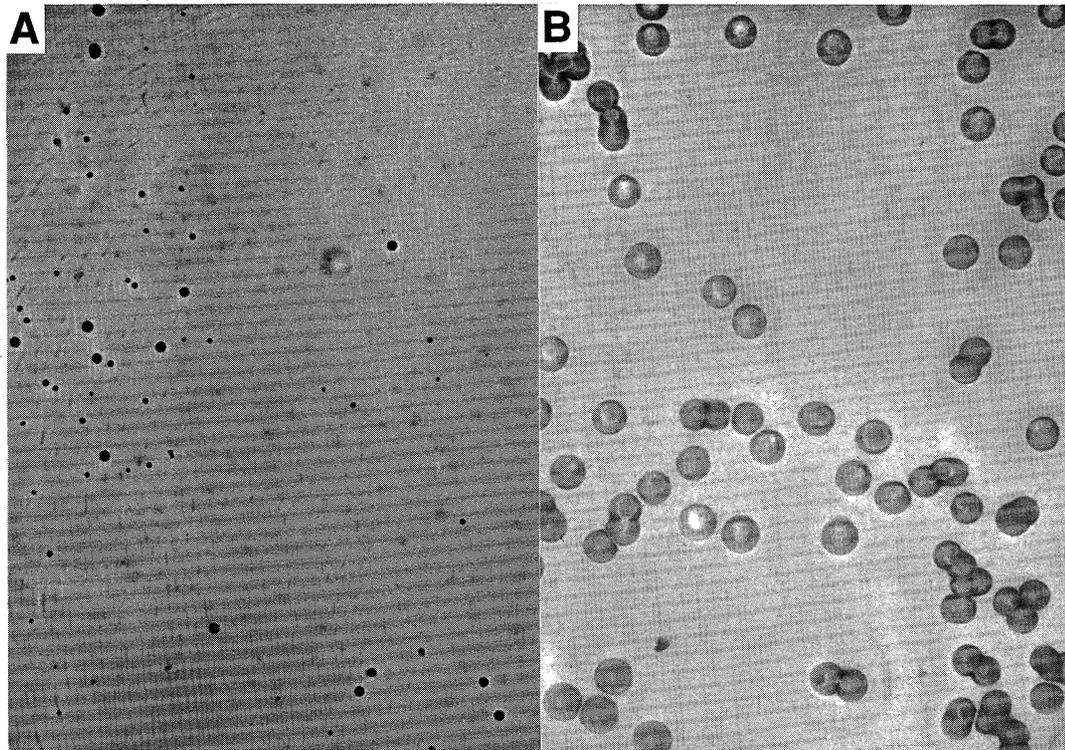


FIG. 1. CV BINDING OF COLONIES OF *Y. ENTEROCOLITICA* GER (SEROTYPE O:3 CELLS GROWN ON CR-BHO FOR 24 h AT 37C

After incubation, CR uptake and Lcr characteristics were observed and then plates were flooded with 8 ml of a 100 $\mu\text{g/ml}$ of CV solution (Bhaduri 1993; Bhaduri *et al.* 1987). (A) Virulent P^+ cells showing pinpoint reddish dark-violet colonies. (b) Avirulent P^- cells showing large white or light pink white colonies. CR-BHO was prepared by adding agarose type V (1.2%) to BHI broth supplemented with 0.1% magnesium chloride and 75 $\mu\text{g/ml}$ of CR (Bhaduri *et al.* 1990, 1991).

were confirmed to be P⁺ by their ability to bind CV. The CR-BHO plates containing colonies could be stored at refrigerated temperature (0–4C) for 24 to 48 h prior to CV binding. The CV binding was not inhibited under these conditions as in the case of BHA reported previously (Bhaduri 1993; Bhaduri *et al.* 1987). This allows recovery of viable P⁺ cells prior to CV binding and HP tests in field laboratory conditions for subsequent investigation. These tests performed with CR-BHO were correlated with the presence of plasmid as well as with a number of virulence properties including mouse virulence as described in previous reports (Bhaduri 1993; Bhaduri *et al.* 1987, 1990, 1991). CR-BHO medium can effectively detect the presence of virulent P⁺ cells in cultures containing predominantly P⁻ cells. Such cultures are not uncommon in clinical laboratories as demonstrated by the FDA investigation in assessing a recent *Yersinia* outbreak in Los Angeles County, CA (Bhaduri 1993; Weagant 1992). Results similar to those obtained with CR-BHO were obtained when CR-agarose-based tryptic soy plating medium was used (data not shown).

The use of CR-agarose-based medium offers distinct advantages over currently available dye binding techniques, since it permits rapid identification and differentiation of P⁺ and P⁻ strains of *Y. enterocolitica* based on multiple plasmid-associated virulence determinants using a single medium. Its use does not require special equipment and can be used to screen large numbers of cultures. Thus, CR-agarose-based medium fulfills four basic requirements for diagnostic tests: speed, accuracy, simplicity and low cost.

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REFERENCES

- BHADURI, S. 1993. Simple and rapid methods for identification of virulent plasmid-bearing clones of *Yersinia enterocolitica*. *J. Rapid Methods Automation Microbiol.* 2, 27–38.
- BHADURI, S., CONWAY, L.K. and LACHICA, R.V. 1987. Assay of crystal violet binding for rapid identification of virulent plasmid-bearing clones of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 25, 1039–1042.
- BHADURI, S. and TURNER-JONES, C. 1993. Effect of anaerobic atmospheres on the stability of the virulence related-characteristics in *Yersinia enterocolitica*. *Food Microbiol.* 10, 239–242.
- BHADURI, S., TURNER-JONES, C. and CONWAY, L.K. 1988. Stability of

- the virulence plasmid in *Yersinia enterocolitica* at elevated temperatures. *Food Microbiol.* 5, 231-233.
- BHADURI, S., TURNER-JONES, C. and LACHICA, R.V. 1991. Convenient agarose medium for the simultaneous determination of low calcium response and Congo red binding by virulent strains of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 29, 2341-2344.
- BHADURI, S., TURNER-JONES, C., TAYLOR, M.M. and LACHICA, R.V. 1990. Simple assay of calcium dependency for virulent plasmid-bearing clones of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 28, 798-800.
- BRUBAKER, R.R. 1991. Factors promoting acute and chronic diseases caused by *Yersiniae*. *Clin. Microbiol. Rev.* 4, 309-324.
- CORNELIS, G., LAROCHE, Y., BALLIGAND, G., SORY, M.P. and WAUTERS, G. 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. *Rev. Infect. Dis.* 9, 64-87.
- DOYLE, M.P. and CLIVER, D.O. 1990. *Yersinia enterocolitica*. In *Foodborne Diseases*, (D.O. Cliver, ed.) pp. 223-228, Academic Press, San Diego, CA.
- FARMER, J.J. III, CARTER, G.P., MILLER, V.L., FALKOW, S. and WACHSMUTH, I.K. 1992. Pyrazinamidase, CR-MOX Agar, Salicin Fermentation-Esculin for identifying pathogenic serotypes of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 30, 2589-2594.
- Food and Drug Admin. 1992. *Bacteriological Analytical Manual*, 7th Ed., pp. 95-109, AOAC International, Arlington, VA.
- KAPPERUD, G. 1991. *Yersinia enterocolitica* in food hygiene. *Int. J. Food Microbiol.* 12, 53-66.
- KOEPPEL, E., MEYER, R., LUETHY, J. and CANDRIAN, U. 1993. Recognition of pathogenic *Yersinia enterocolitica* by crystal violet binding and polymerase chain reaction. *Lett. Appl. Microbiol.* 17, 231-234.
- KWAGA, J.K.P. and IVERSEN, J.O. 1991. Laboratory investigation of virulence among strains of *Yersinia enterocolitica* and related species from pigs and pork products. *Can. J. Microbiol.* 38, 92-97.
- LACHICA, R.V. and ZINK, D.L. 1984. Determination of plasmid-associated hydrophobicity for *Yersinia enterocolitica*. *J. Clin. Microbiol.* 19, 660-663.
- PORTNOY, D.A. and MARTINEZ, R.J. 1985. Role of a plasmid in the pathogenicity of *Yersinia* species. In *Genetic Approaches to Microbial Pathogenicity*, (W. Goebel, ed.) pp. 29-51, Springer-Verlag, New York.
- ROBINS-BROWNE, R.M., MILIOTIS, M.D., CIANCIOSI, S., MILLER, V.L., FALKOW, S. and MORRIS, J.G., JR. 1989. Evaluation of DNA colony hybridization and other techniques for detection of virulence in *Yersinia* species. *J. Clin. Microbiol.* 27, 644-650.
- WEAGANT, S.D. 1992. Personal communication. FDA, Bothell, WA.