DIRECT ISOLATION OF PLASMID-BEARING VIRULENT *YERSINIA ENTEROCOLITICA* FROM PORK SAMPLES

SAUMYA BHADURI, BRYAN COTTRELL and ARTHUR J. MILLER

Microbial Food Safety Research Unit, Eastern Regional Research Center, USDA, ARS, 600 East Mermaid Lane, Wyndmoor, PA 19038

INTRODUCTION

Pigs and pork meat have been identified as important reservoirs of *Yersinia enterocolitica*, including those serotypes which have been associated with human illness (Ravagnan and Chiesa, 1995). Strains of all serotypes implicated in human disease harbor a plasmid of 70 to 75 kbp, which is directly involved in the virulence of *Y. enterocolitica* (YEP^+) (Kapperud 1991; Ravagnan and Chiesa 1995). A wide variety of methods for the isolation of YEP^+ from pork have been described (Ravagnan and Chiesa, 1995). The efficiency of enrichment techniques for YEP^+ varies greatly with the serotype. No single isolation procedure has been described for simultaneous isolation and detection of YEP^+ from pork samples. The unstable nature of the virulence plasmid (Bhaduri, 1993; Ravagnan and Chiesa, 1995) complicates the isolation of YEP^+ due to the overgrowth of plasmidless avirulent cells in the population leading to a completely avirulent culture. Since the population of YEP^+ in pork meat samples is usually low, and the natural microflora tend to suppress the growth of YEP^+ (Ravagnan and Chiesa, 1995), isolation methods usually involve enrichment followed by plating onto selective media. Methods described in the literature do not confirm *Y. enterocolitica* presumptives as YEP^+ strains. Therefore, the objective of this study was to directly detect and isolate various strains of YEP^+ serotypes in pork samples potentially harboring this organism by using Congo red (CR) binding and low calcium response (Lcr) tests.

MATERIALS AND METHODS

Bacteria and preparation of media

A number of YEP^+ strains representing O:3; O:8; O:TACOMA; O:5, O:27; and O:13 serotypes were used in this study (Bhaduri et al. 1997). Modified trypticase soy broth (MTSB) (Difco Laboratories, Detroit, Mich.) containing bile salts #3 (Difco) was prepared as described by Bhaduri et al. (Bhaduri et al. 1997). Brain heart infusion (BHI) (Difco) broth, calcium-adequate (1,500 μM) brain heart infusion agar (BHA) (Difco), and low-calcium (238 μM) CR (Sigma Chemical Co., St. Louis, Mo) BHI agarose (Gibco BRL, Gaithersburg, Md) (CR-BHO) were prepared as described previously (Bhaduri et al. 1993).

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.

Food samples

Pork chop and ground pork were purchased from a local super market and stored at 4°C. Porcine tongues were collected from Hatfield Quality Meats, Hatfield, PA and processed as described previously (Bhaduri et al. 1997).

Enrichment

Swabs of 10 cm^2. pork chop and ground pork medallions spiked with appropriate dilutions of YEP^+ cultures were placed in sterile Whirl Pak bags containing 90 ml of MTSB and allowed to stand at room temperature for 5 min. The porcine tongues were swabbed individually over the whole surface and treated as described above. The enrichment bags were placed in a shaking incubator (100 rpm) at 12°C for 24 h. Irgasan (Ciba-Geigy Corp., Greensboro, NC) was then added at a final concentration of 4 μg/ml (Bhaduri et al. 1997) and reincubated at 12°C for another 24 h.

Detection, isolation, and virulence of YEP^+ colones

Selectively enriched samples were diluted and plated on BHA for total CFU counts and on CR-BHO for direct detection and isolation. All plates were incubated at 37°C for 24 h. Pathogenic YEP^+ strains appeared as red pinpoint colonies (CR^+) on CR-BHO (Bhaduri et al. 1993). The pigmentation is due to CR binding and the pinpoint colonial morphology is due to a Lcr (Bhaduri et al. 1993). The simultaneous expression of these two plasmid-associated phenotypes identifies isolates YEP^+ strains. The identification of the CR^+ colonies was further confirmed by multiplex PCR using chromosomal *ail* gene (attachment-invasion locus) and *virF* gene (transcriptional activator for the expression of plasmid-encoded outer membrane protein *yop* 51) from the virulence plasmid (Bhaduri et al. 1997). The CR^+ colonies were cultured in BHI broth at 28°C for 24 h and stored in 10% glycerol at -70°C. The expression of the virulence plasmid in the recovered YEP^+ strains was verified by colonial morphology, crystal violet binding, Lcr, CR binding, hydrophobicity, autoagglutination, and mouse virulence tests as described previously (Bhaduri et al. 1993).

RESULTS AND DISCUSSION

Studies were done to determine the usefulness of swabbing technique for the recovery of YEP^+ strains from pork samples. Medallions of pork chop and ground pork artificially contaminated with various concentrations of YEP^+ strain GER (serotype O: 3) were used to standardize optimal conditions for the method. It was found that addition of Irgasan at 24 h (day 2) gave the best recovery of YEP^+ colonies as reported previously (Bhaduri et al. 1997). The YEP^+ colonies from artificially contaminated pork chop (Fig. 1A) and ground pork (Fig. 1B) appeared as CR^+ colonies (red pin point) respectively (day 4). Thus, YEP^+ strains from each food sample were identified and isolated directly by CR binding and Lcr techniques (Bhaduri et al 1993). Identification of YEP^+ strains on CR-BHO allows the recovery of *Y. enterocolitica* colonies harboring the virulence plasmid (Bhaduri et al. 1993). The CR^+ clones were further confirmed as YEP^+ strains by multiplex PCR with primers directed at the chromosomal *ail* and *virF* which amplified a 170-bp product from the chromosome and 591-bp product from the virulence plasmid, respectively (Bhaduri et al. 1997) (data not shown).

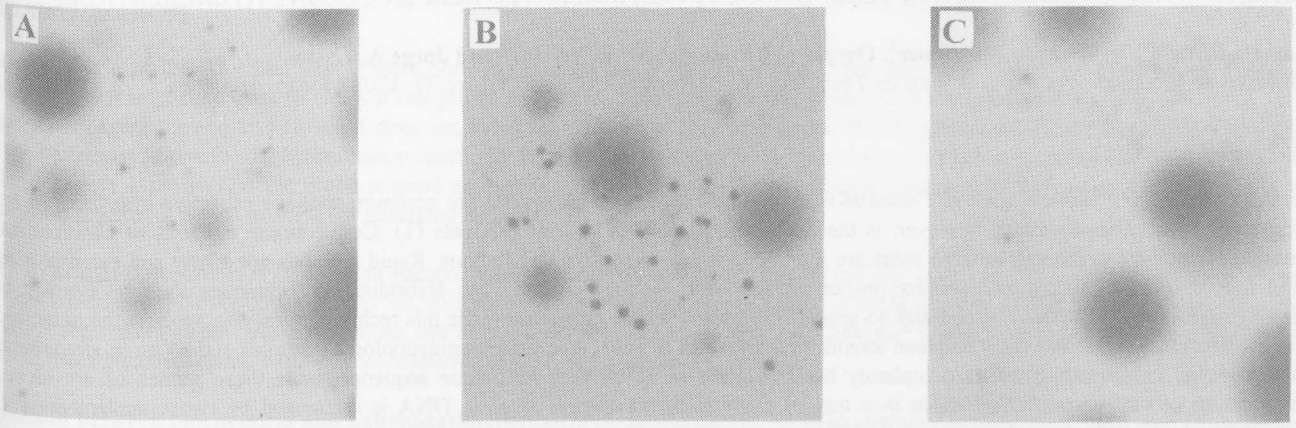

Fig. 1. Recovery of YEP$^+$ strains as red pin point colonies on CR-BHO from artificially contaminated pork chop (A), ground pork (B), and naturally contaminated porcine tongue (C).

This method is completed in 4 days from sample enrichment to detection and isolation including confirmation by multiplex PCR and can recover YEP$^+$ strains in pork chop and ground pork spiked with as low as 0.5 CFU cm^2 (Table 1). This technique has been successfully applied in the recovery of different YEP$^+$ strains of five serotypes including O:3 (five strains), O:8 (five strains), O:TACOMA (four strains), O:5, O:27 (four strains), and O:13 (three strains) from artificially contaminated pork samples as described above. The successful isolation of YEP$^+$ strains from naturally contaminated porcine tongue verified the effectiveness of this method. Of 17 tongues analyzed, seven were CR$^+$ positive (Fig. 1C) as YEP$^+$ strains (~41%) by both CR binding and Lcr. PCR analysis confirmed the presence of a 170-bp product from the chromosome and a 591-bp product from the virulence plasmid (Bhaduri et al. 1997) (data not shown). All isolates from tongue were serotype O:3 as reported previously (Bhaduri et al. 1997). The virulence of YEP$^+$ strains recovered from both artificially contaminated pork chop, ground pork, and naturally contaminated tongues was confirmed by a number of plasmid-associated virulence characteristics including colonial morphology, crystal violet binding, Lcr, CR uptake, hydrophobicity, autoagglutination, and mouse virulence test results. These results showed that the organism retained the virulence plasmid, phenotypic characteristics and pathogenicity after its recovery from pork chop, ground pork, and porcine tongue.

Table 1. Sensitivity of recovery of YEP$^+$ strain from artificially contaminated pork chop and ground pork.

Concentration of YEP$^+$ strain (CFU/cm^2)	YEP$^+$ strain confirmation[a]	
	Pork Chop	Ground Pork
10	+	+
1	+	+
0.5	+	+
0.1	-	-

[a]Confirmed by CR binding (YEP$^+$cells appeared as red pin point colonies on (CR-BHO) and PCR on day 4. +, present; -, absent.

The method has the following advantages:(i) use of one single medium (CR-BHO) for both direct detection and isolation eliminates the presumptive isolation step and preserves the virulence plasmid; (ii) reduces by 2 days the recovery method described in our original report (Bhaduri et al. 1997). Thus, this procedure is a practical alternative to many other recovery methods which require significantly more time for completion.

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