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Direct Detection and Isolation of Plasmid-Bearing Virulent Serotypes of *Yersinia enterocolitica* from Various Foods

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A procedure was developed for direct detection, isolation, and maintenance of plasmid-bearing virulent serotypes of *Yersinia enterocolitica* from different food sources. Plasmid-bearing virulent strains of *Y. enterocolitica* representing five serotypes were simultaneously detected and isolated from enriched swab samples of artificially contaminated pork chops, ground pork, cheese, and zucchini, using Congo red binding and low-calcium-response tests. The method was also effective in isolating plasmid-bearing virulent strains of *Y. enterocolitica* from naturally contaminated porcine tongues. Virulence of the strains isolated from these foods was confirmed by PCR, the expression of plasmid-associated phenotypes, and mouse pathogenicity.

Yersinia enterocolitica is a food-borne pathogen of significance to humans (9, 10, 13, 14). Strains of all serotypes implicated in human disease harbor a virulence-associated plasmid of 70 to 75 kbp (9, 10, 13).

Common food vehicles in outbreaks of yersiniosis are meat (particularly pork), milk, dairy products, powdered milk, cheese, tofu, and raw vegetables (9, 10, 13, 14). A number of methods for the isolation of *Y. enterocolitica* from food have been described (13–15). The efficiency of *Y. enterocolitica* recovery varies with serotype and depends on the type of food being tested. Different isolation procedures have been described to recover the full range of plasmid-bearing virulent serotypes of *Y. enterocolitica* (YEP^+) from a variety of foods (13). Recently, we developed a rapid procedure for the selective enrichment, isolation, identification, and maintenance of YEP^+ strains from pork samples (4). Despite the advantages of the method compared to older procedures, there were several areas where additional improvements could be realized. Food matrices can inhibit the enrichment of YEP^+ strains (unpublished data). The initial isolation of presumptive *Y. enterocolitica* from enriched samples on MacConkey agar adds an extra plating step, and the picking of presumptive *Y. enterocolitica* requires skilled recognition and handling of the colonies (4). The unstable nature of the virulence plasmid (6, 9, 10, 13) complicates the detection of YEP^+ strains, since isolation steps may lead to plasmid loss and the associated phenotypic characteristics for colony differentiation. Finally, the handling and preparation of food samples is time-consuming.

Food surfaces are often the primary site of bacterial contamination. Nondestructive swabbing has been used widely as a surface sampling procedure for the isolation and detection of a wide variety of food-borne pathogens from meat (8). No single procedure has been described for simultaneous isolation and detection of YEP^+ strains from a variety of foods. In the present study, we attempted to directly detect and isolate various strains of YEP^+ serotypes by enriching swabs of artificially contaminated pork chops, ground pork, cheese, and zucchini in a single enrichment medium and applying Congo red (CR) binding, low calcium response (Lcr), and PCR for confirmation.

Bacteria and preparation of media. Different YEP^+ strains representing O:3, O:8, O:TACOMA, O:5,O:27, and O:13 serotypes used in this study were kindly provided by S. W. Weagant, Food and Drug Administration, Seattle District Laboratory, Bothell, Wash. These serotypes were clinical isolates and confirmed as YEP^+ strains by the Food and Drug Administration and our laboratory. A detailed description of preparation of inocula, incubation conditions, and characterization of the strains is given elsewhere (1, 4). Modified trypticase soy broth (MTSB) (Difco Laboratories, Detroit, Mich.) containing 0.2% bile salts no. 3 (Difco) was prepared as described by Bhaduri et al. (4). 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 (3, 6, 7).

Food samples. Pork chops, ground pork, cheese (Kraft cheese singles), and zucchini were purchased from a local market and stored at 4°C. Medallions of each food type were prepared using a 10-cm^2 coring tool. Porcine tongues were collected from Hatfield Quality Meats, Hatfield, Pa., and processed as described previously (4).

Inoculation of samples. YEP^+ cultures were grown in BHI broth for 18 h at 25°C with shaking to a population density of approximately 10^9 CFU/ml (4). Dilutions containing 1,000, 100, 50, and 10 CFU of YEP^+ strains per ml were pipetted (100 μl) over the food surfaces giving 10, 1, 0.5, and 0.1 CFU/cm^2. To simulate surface contamination, the samples were allowed to stand for 15 min.

Swabbing. The medallions were sampled by swabbing the surface with a sterile 5 by 5 by 1.25-cm cellulose sponge moistened in 10 ml of MTSB. Each swab was placed in a sterile Whirl Pak bag containing 90 ml of MTSB and allowed to stand at room temperature for 5 min. Porcine tongues were surface swabbed and treated as described above.

Enrichment. The enrichment bags were placed in a shaking incubator (100 rpm) at 12°C for 24 h. Irgasan (Ciba-Geigy Corp., Greensboro, N.C.) was then added to yield a final concentration of 4 μg/ml (4), and the bags were reincubated at 12°C for another 24 h.

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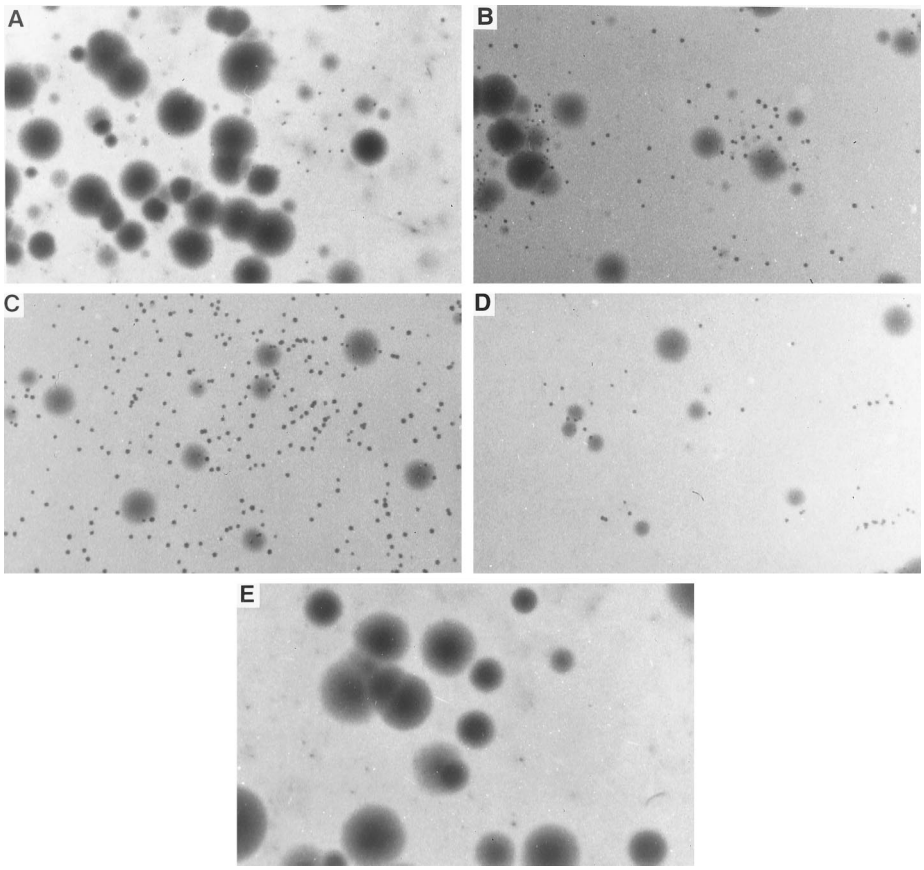

FIG. 1. Recovery of YEP$^+$ strains as red pinpoint colonies on CR-BHO from artificially contaminated pork chops (A), ground pork (B), cheese (C), zucchini (D), and naturally contaminated porcine tongue (E).

Sampling of enriched cultures. Sampling of the bags was performed at 48 and 72 h of total enrichment time. Each enrichment bag was mixed immediately prior to sampling (4).

Direct detection, isolation, and virulence of YEP$^+$ clones. Selectively enriched samples were diluted and plated on CR-BHO. All plates were incubated at 37°C for 24 h. Pathogenic YEP$^+$ strains appear as red pinpoint colonies (CR$^+$) on CR-BHO (6, 7). The pigmentation is due to CR binding, and the pinpoint morphology is due to an Lcr encoded by the virulence plasmid present in YEP$^+$ strains (6, 7). Thus, the simultaneous expression of these two plasmid-associated phenotypes identifies YEP$^+$ clones. The identification of the CR$^+$ colonies was confirmed by multiplex PCR using the 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 (5). The CR$^+$ colonies were stored as described by Bhaduri et al. (4). The presence 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 (2, 3, 6, 7, 11, 12).

The conditions for the direct detection, isolation, and maintenance of YEP$^+$ serotypes are based on single-medium culture at 12°C. Enrichment of swabs was optimized using pork chops, ground pork, cheese, and zucchini artificially contaminated with various concentrations of YEP$^+$ strain GER (serotype O:3). It was determined that Irgasan should be added 24 h (day 2) after the initial enrichment of the YEP$^+$ strains at 12°C to reduce the inhibitory effect of the antibiotic on its growth. The addition of Irgasan after 24 h (day 2) and incubation for an additional 24 h (day 3) at 12°C allowed the growth of YEP$^+$ strains while effectively inhibiting growth of competing microflora. Thus, YEP$^+$ strains were able to grow to a detectable level even in the presence of competing microflora. It was also determined that sampling should be done at 48 h of total incubation to avoid sampling after competing microflora begin to predominate. This technique enhances isolation of YEP$^+$ strains in the presence of competing microflora through the selection of optimal incubation temperature, sampling schedule, and timing for the addition of Irgasan. Thus, YEP$^+$ strains could be detected when directly plated on CR-BHO after 48 h of total incubation at 12°C (day 3). This modified protocol reduced the time of our original method (4) by 2 days by eliminating 1 day of enrichment and 1 day for plating on MacConkey agar. The YEP$^+$ colonies from artificially contaminated pork chop (Fig. 1A), ground pork (Fig. 1B), cheese (Fig. 1C), and zucchini (Fig. 1D) all appeared as CR$^+$ (red pinpoint) colonies (day 4). Thus, YEP$^+$ strains from each food sample were identified as harboring the virulence plasmid. The CR$^+$ clones were further confirmed as YEP$^+$ strains by multiplex PCR. Primers amplified a 170-bp product from the chromosome (*ail* gene) and 591-bp product from the virulence plasmid (*virF* gene) (Fig. 2, pork chops, lanes 2 to 4; ground pork, lanes 8 to 10; cheese, lanes 14 to 16; and zucchini, lanes 20 to 22). The presence of the *ail* gene specifically differentiates YEP$^+$ strains from environmental *Y. enterocolitica* and *Y. pseudotuberculosis* lacking this gene (5). The presence of the *virF* gene demonstrates the presence of the virulence plasmid which con-

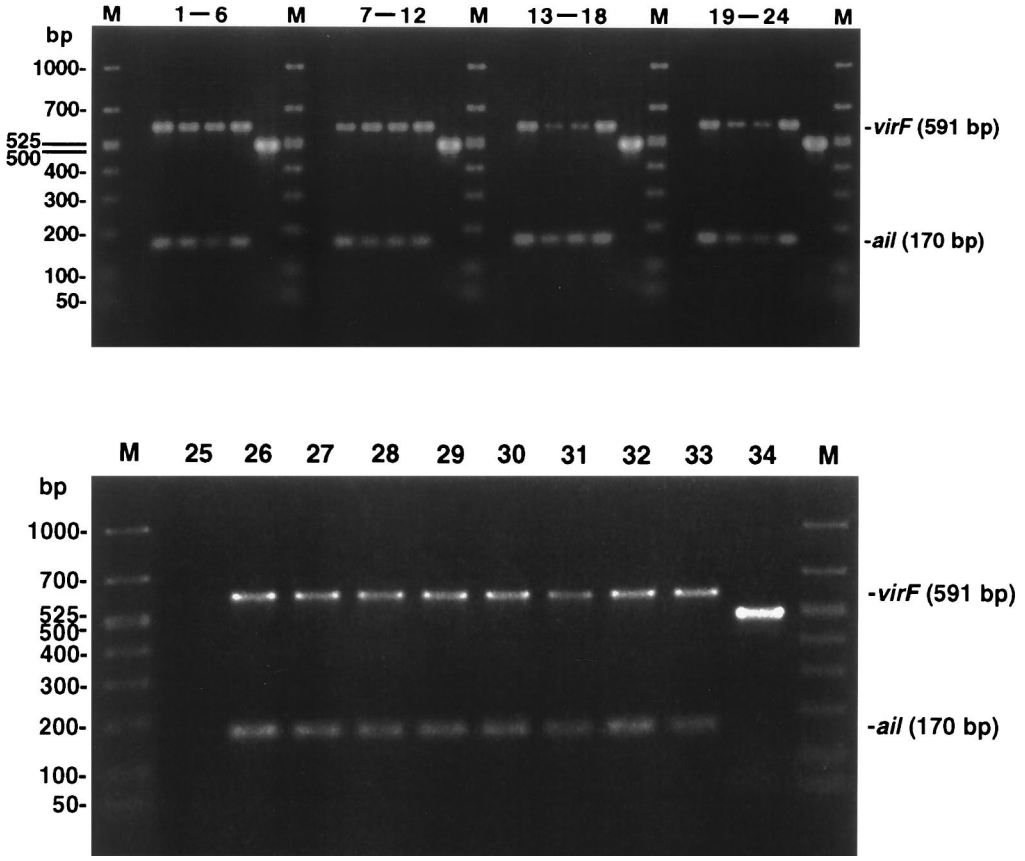

FIG. 2. Confirmation of CR^+ clones isolated from various artificially contaminated foods and from naturally contaminated porcine tongues as YEP^+ strains by multiplex PCR using the chromosomal *ail* gene and the *virF* gene from the virulence plasmid. Lane M, 50- to 1,000-bp ladder marker; lanes 1, 7, 13, 19, and 25, negative control with no template, CR^+ colony showing the presence of 170- and 591-bp products with mixture of both *ail* and *virF* primers from the chromosome and virulence plasmid, respectively, isolated from pork chops (lanes 2 to 4), ground pork (lanes 8 to 10), cheese, (lanes 14 to 16), zucchini (lanes 20 to 22), and porcine tongues (lanes 26 to 32). Lanes 5, 11, 17, 23, and 33, positive control with purified DNA from YEP^+ strain showing the presence of 170- and 591-bp products with mixture of both *ail* and *virF* primers from the chromosome and virulence plasmid, respectively; lanes 6, 12, 15, 24, and 34, positive control for PCR assay with λ as DNA template.

fers the plasmid-associated phenotypes. Thus, the YEP^+ virulent strains were identified by both virulence plasmid-associated phenotypic expression and the presence of specific virulence genes.

This method can be completed in a minimum of 4 days beginning with sample enrichment and concluding with confirmation by multiplex PCR. A 4-day recovery was achieved for all samples at initial inoculum levels of 10 and 1 CFU/cm^2. YEP^+ strains could also be recovered in 4 days from pork chops and ground pork at an initial inoculum level of 0.5 CFU/cm^2, while cheese and zucchini at this level required an additional day of enrichment for positive identification. YEP^+ strains could not be recovered from any of the samples at an initial contamination level of 0.1 CFU/cm^2 regardless of the length of enrichment. 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 the foods mentioned. The successful isolation of YEP^+ strains from naturally contaminated porcine tongue verified the effectiveness of this method. Of 17 tongues analyzed, 7 (~41%) were positive for YEP^+ by both CR binding and Lcr (Fig. 1E). The percentage recovery of YEP^+ strains is comparable to that of the original method (4). PCR analysis confirmed the presence of a 170-bp product from the chromosome and a 591-bp product from the virulence plasmid (Fig. 2, lanes 26 to 32). All isolates from tongue were serotype O:3. The virulence of YEP^+ strains recovered from both artificially contaminated food samples and naturally contaminated tongues was confirmed by plasmid-associated virulence characteristics and mouse virulence testing. These results demonstrate that YEP^+ strains recovered using this method retain the virulence plasmid, phenotypic characteristics, and pathogenicity after isolation from pork chops, ground pork, cheese, zucchini, and porcine tongue.

Conclusion. This method has the following advantages: (i) it uses a single enrichment medium for a range of serotypes including a large number of different strains from a variety of foods, (ii) it eliminates 1 day of enrichment and another for the presumptive isolation, (iii) it uses a single medium (CR-BHO) for direct detection and isolation, and (iv) it preserves the virulence plasmid. This procedure is a practical alternative to many other recovery methods which require significantly more time for completion.

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