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Use of a Single Procedure for Selective Enrichment, Isolation, and Identification of Plasmid-Bearing Virulent *Yersinia enterocolitica* of Various Serotypes from Pork Samples

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Many selective enrichment methods for the isolation of *Yersinia enterocolitica* from foods have been described. However, no single isolation procedure has been described for the recovery and identification of various plasmid-bearing serotypes. A single improved procedure for selective enrichment, isolation, identification, and maintenance of plasmid-bearing virulent serotypes of *Y. enterocolitica* from pork samples was developed. Enrichment at 12°C in Trypticase soy broth containing yeast extract, bile salts, and Irgasan was found to be an efficient medium for the recovery of plasmid-bearing virulent strains of *Y. enterocolitica* representing O:3; O:8; O:TACOMA; O:5, O:27; and O:13 serotypes. MacConkey agar proved to be a reliable medium for the isolation of presumptive colonies, which were subsequently confirmed as plasmid-bearing virulent strains by Congo red binding and low calcium response. Further confirmation by multiplex PCR employed primers directed at the chromosomal *ail* and plasmid-borne *virF* genes, which are present only in pathogenic strains. The method was applied to pig slaughterhouse samples and was effective in isolating plasmid-bearing virulent strains of *Y. enterocolitica* from naturally contaminated porcine tongues. Strains isolated from ground pork and tongue expressed plasmid-associated phenotypes and mouse pathogenicity.

The association of human illness with the consumption of food contaminated with *Yersinia enterocolitica* is well documented (10, 12, 13, 21). Pigs and pork have been identified as important reservoirs of *Yersinia*, including those species and serotypes causing human illness (13, 21). Strains of all serotypes implicated in human disease harbor a plasmid of 70 to 75 kbp which is directly involved in the virulence of this bacterium (10, 13, 21).

Since yersiniae can grow at low temperatures, even refrigerated foods are potential vehicles for the growth of these organisms (10, 12). A wide variety of methods for the isolation of *Y. enterocolitica* from foods have been described (21, 24). Traditional methods employ the use of prolonged enrichment at refrigeration temperatures to take advantage of the psychrotrophic nature of *Y. enterocolitica* and to suppress the growth of background flora. Due to the extended time period needed for this type of method, efforts have been made to devise selective enrichment techniques employing shorter incubation times and higher temperature, thus making them more practical for routine use (10, 12, 13, 23). However, high levels of indigenous microorganisms can overgrow and mask the presence of *Y. enterocolitica* strains (12, 13, 21, 23), including nonpathogenic strains which are frequently isolated from food samples and are atypical in some biochemical reactions (22). It also appears that the efficiency of *Y. enterocolitica* enrichment techniques varies greatly with serotype; no single procedure has been shown to recover a broad spectrum of pathogenic *Y. enterocolitica* strains (21). The most rapid procedure available for the isolation of a wide spectrum of *Y. enterocolitica* strains does not include the identification of isolates as plasmid-bearing virulent strains (23). The unstable nature of the virulence plasmid (6, 10, 12, 13, 21) complicates the isolation of plasmid-bearing virulent *Y. enterocolitica* by causing the overgrowth of virulent cells by plasmidless revertants, eventually leading to a completely avirulent culture.

Since the population of *Y. enterocolitica* in pork meat samples is usually low and natural microflora tend to suppress the growth of this organism (18), isolation methods usually involve enrichment followed by plating onto selective media. Methods described in the literature do not treat confirmation of virulence in presumptive or known *Y. enterocolitica* isolates recovered from selective agars as an integral part of the detection method. Therefore, the objective of this study was to develop a single procedure for selective enrichment, isolation, identification, and maintenance of plasmid-bearing pathogenic serotypes of *Y. enterocolitica* from pork samples potentially harboring this organism.

MATERIALS AND METHODS

Bacteria. A number of plasmid-bearing virulent strains of *Y. enterocolitica* (YEP^+) representing O:3; O:8; O:TACOMA; O:5, O:27; and O:13 serotypes obtained from different sources were used in this study. A detailed description of the strains, sources, preparation of inocula, and incubation conditions is given elsewhere (1).

Preparation of media. Modified Trypticase soy broth (MTSB) was prepared as described by Toora et al. (23) with modification. First, 30 g of TSB (Difco Laboratories, Detroit, Mich.), 2.5 g of yeast extract (Difco), and 2 g of bile salts #3 (Difco) were dissolved in 1,000 ml of double-distilled, deionized water. The pH of the medium was adjusted to 7.6 with 2 M NaOH solution before sterilization. Cefsulodin-Irgasan-Novobiocin (CIN) (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) agar was prepared as recommended by the manufacturer. MacConkey (MAC) agar (Difco), brain heart infusion (BHI) (Difco) broth and brain heart infusion agar (BHA) (Difco) were prepared as recommended by the supplier. Since the concentration of calcium is high in BHA (1,500 μM), it was designated and used as a calcium-adequate medium (7). Low-calcium (238 μM) Congo red (CR) (Sigma Chemical Co., St. Louis, Mo.)-brain heart infusion agarose (CR-BHO) was prepared by adding agarose (Gibco BRL, Gaithersburg, Md.) as the gelling agent to a final concentration of 1.2% to BHI broth supplemented with 0.1% magnesium chloride (6, 7).

Pork samples. Ground pork from a local market was portioned in 10-g aliquot packets and stored at −70°C. Tongue and head meat samples were collected from Hatfield Quality Meats, Hatfield, Pa., and used fresh. Samples were placed in separate sterile Whirl Pak bags (Nasco, Fort Atkinson, Wis.), transported to the laboratory on ice, and processed within 2 h of collection.

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Preparation of inocula. 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. YEP^+ strain GER (serotype O:3) was initially used for standardization in this procedure. The cultures were appropriately diluted prior to inoculating the ground pork as described below.

Sample handling and preparation. Ground pork (10 g) was thawed at room temperature and placed in a sterile Whirl Pak bag containing 10 ml of MTSB and dispersed into a slurry. Appropriate dilutions of YEP^+ strains were added, dispersed into the ground pork, and allowed to stand for 5 min. In the case of tongue and head meat samples, preparation of each sample was done individually by using a sterile cutting board and knife. For tongue samples, a cross section was cut at the center of the tongue. Skin surface samples were also taken. The tongue and head meat samples were finely minced and mixed. A 10-g aliquot of each sample from ground pork, tongue, or head meat was placed in a sterile Whirl Pak bag. Then, 10 ml of MTSB broth was added to each sample, and the samples were allowed to stand for 5 min. An additional 80 ml of MTSB was added to bring the total volume to 10 times the pork mass and was thoroughly mixed into the pork.

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 at a final concentration of 4 μg/ml, and the bags were reincubated at 12°C for another 48 h. Cold enrichment (12, 21) of the pork samples at 4°C in MTSB for 4 weeks was used as a control procedure.

Sampling of enriched cultures. Sampling from the bags was started after 72 h of total enrichment time. Each enrichment bag was mixed immediately before sampling.

Isolation of presumptive *Y. enterocolitica* colonies. Selectively enriched samples were diluted and plated on BHA for CFU counts and on MAC and CIN agars for presumptive isolation. All plates were incubated at 28°C for a maximum of 24 h.

Picking of presumptive *Y. enterocolitica* colonies from MAC and CIN agars. Presumptive *Y. enterocolitica* colonies were detected after 19 to 20 h of incubation. Plates were screened by using a dissecting microscope at 7× magnification to select those colonies measuring 0.2 to 0.25 mm in diameter. The presumptive colonies appear flat, translucent, and colorless (*Yersinia* does not ferment lactose) and are the minority of colonies present. These colonies were marked and further incubated at 28°C for 2 to 3 h or until there was sufficient growth to pick with a needle. Individual disposable sterile needles were dipped in sterile water to pick up single colonies; water allowed easier diffusion of the colonies for transfer to CR-BHO. From CIN agar, small (diameter, 1 to 2 mm) colonies having a deep red center and sharp border and surrounded by a clear colorless zone with entire edge were transferred onto CR-BHO as described above. These plates were incubated for 18 to 24 h at 37°C.

Identification of virulent plasmid-bearing clones of *Y. enterocolitica*. (i) Congo red binding technique. Pathogenic YEP^+ strains appear as red pinpoint colonies (CR^+) on CR-BHO. The pigmentation is due to CR binding, and the pinpoint colonial morphology is due to a low-calcium response (Lcr) (6, 7). The simultaneous expression of these two plasmid-associated phenotypes identifies YEP^+ strains. Another characteristic feature of CR binding is the appearance of an occasional white border around the red pinpoint center which becomes more pronounced with prolonged incubation at 37°C and can be used as an additional confirmation of virulent YEP^+ strains (6).

(ii) Confirmation of CR^+ clones by multiplex PCR. The identification of the CR^+ colonies was further 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, 8, 20). The primer pairs (5′-ACTCGATGATAACTGGGGAG-3′ and 5′-CCCCCAGTAATCCATAAAGG-3′) for detection of the *ail* gene (664- to 833-nucleotide region) amplified a 170-bp DNA fragment from the chromosome (5). The primer pairs (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) for detection of the *virF* gene (430- to 1,020-nucleotide region) amplified a 591-bp product from the virulence plasmid (5). A mixture of primers for both the *ail* and *virF* genes was used for detection of both genes in a multiplex reaction (5). The oligonucleotide primers utilized in this study were synthesized by the Appligene Company (Pleasanton, Calif.). PCR was performed as previously reported (5). Purified chromosomal and plasmid DNA from YEP^+ cells was used as a positive control (4, 5). A negative control with all of the reaction components except template DNA was included with each test run. After PCR amplification, 5 μl of stop solution (50% glycerol, 0.02% bromophenol blue, 60 mM EDTA, pH 8.0) was added and 5 μl of each PCR product was analyzed by electrophoresis on a 2.0% agarose gel at a constant voltage of 80 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 90 min (4, 5). The amplified DNA fragments were stained with ethidium bromide (0.5 μg per ml) and visualized with UV transillumination at 302 nm (4, 5).

Storage of YEP^+ strains recovered from ground pork and tongue. CR^+ colonies were picked with sterile needles, avoiding colonies that showed white YEP^- revertants, and were cultured in 5 ml of BHI broth for 18 to 24 h at 28°C. The cells were harvested and washed with 5 ml of BHI broth. The pellet was resuspended in 2.5 ml of BHI broth and 2.5 ml of BHI broth–20% glycerol to give a final glycerol concentration of 10%. Then, 200-μl portions were aliquoted in cryogenic vials. The cells were allowed to equilibrate for 15 min after the addition of the glycerol. Vials were stored at −70°C.

Determination of in vitro properties associated with the virulence of YEP^+ strains after recovery from ground pork and tongue. The expression of the virulence plasmid in the recovered YEP^+ strains was verified by colonial morphology, crystal violet (CV) binding, Lcr, CR binding, hydrophobicity (HP) and autoagglutination (AA) by using the tests described below. The colony morphology of bacteria was tested by growth on calcium-adequate BHA. Low-calcium CR-BHO medium was used for CR binding and Lcr. The CV binding assay was done on calcium-adequate BHA. Detailed descriptions of these assay conditions are given elsewhere (3, 6, 7). HP was examined by using latex particle agglutination (LPA) (14). AA was determined as previously described (15) with Eagle minimal essential medium supplemented with 10% fetal bovine serum. The ability of strains of *Y. enterocolitica* to express these plasmid-associated virulence characteristics was tested as follows. YEP^+ and YEP^- strains were grown in BHI broth for 18 h at 25°C with shaking to a population density of approximately 10^9 CFU/ml. The cells were diluted to 10^3 cells per ml, and 100 μl was surface plated onto BHA and CR-BHO plates. Plates were incubated at 37°C for 24 h and observed for the phenotypic expression of the virulence plasmid.

Serotyping of YEP^+ strains recovered from tongues. The serotyping of YEP^+ strains from tongues was based on heat-stable O antigens and was kindly performed by J. Michael Janda (chief of the Enteric Special Pathogen Section, Microbial Diseases Laboratory, State of California-Department of Health Services, Berkeley).

Mouse virulence assay. Swiss Webster albino male mice (15 to 20 g each) were used to determine the virulence of the strains. The mice were pretreated with 5 mg of iron-dextran (Imferon; Fermenta Animal Health Company, Kansas City, Mo.) and 5 mg of desferroxamine (Desferal; CIBA-GEIGY Corp. Suffern, N.Y.), infected orally, and examined daily for diarrhea (2). The protocol for the mouse virulence assay was approved by the Institutional Animal Care & Use Committee.

RESULTS AND DISCUSSION

Enrichment methods for *Y. enterocolitica* reported in the literature permit recovery, but they do not attempt to specifically confirm the presence of plasmid-bearing virulent strains. In this work, a single procedure using enrichment at 12°C was developed for isolation, identification, and maintenance of YEP^+ strains from pork samples. Ground pork artificially contaminated with various concentrations of YEP^+ strain GER (serotype O:3) was used to standardize optimal conditions for the method. The addition of Irgasan played a critical role in the enrichment of YEP^+. We were not successful in recovering YEP^+ colonies from ground pork samples by using an MTSB enrichment that includes Irgasan in the initial medium as described by Toora et al. (23). Since Irgasan suppresses the growth of pure YEP^+ cultures grown in MTSB when added at the onset of growth and does not when added after the lag phase, we eliminated it from the initial enrichment medium. In tests to recover YEP^+ colonies from ground pork, Irgasan was added at 24, 48, and 72 h. It was found that addition of Irgasan at 24 h gave the best recovery of YEP^+ colonies. This timing may reduce the inhibitory effect of the antibiotic (9). The addition of Irgasan after 24 h (day 2) and incubation for an additional 48 h (day 4) at 12°C allowed *Yersinia* strains to grow to detectable levels even in the presence of competing microflora. It was also determined that sampling should be done at 48 h of incubation to allow any *Yersinia* present time to reach detectable levels and 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 incubation temperature, sampling schedule, and timing of antibiotic addition.

Both MAC and CIN agars were used for presumptive isolation of YEP^+ strains. Presumptive colonies were first identified as YEP^+ by CR binding and Lcr techniques (6, 7). The identification of YEP^+ strains on CR-BHO allows the recovery of *Y. enterocolitica* colonies harboring the virulence plasmid (6). Presumptive colonies identified as CR^+ clones were successfully isolated from MAC agar, whereas, all typical presumptive colonies from CIN agar tested negative. Thus, CIN

TABLE 1. Sensitivity of recovery of YEP$^+$ strain from artificially contaminated ground pork

YEP$^+$ strain (CFU/g)	Initial ratio (YEP$^+$:BKG[a])	YEP$^+$ strain confirmation[b]
25,000	1:40–1:400	+
9,371	1:100–1:1,000	+
5,160	1:200–1:2,000	+
3,000	1:300–1:3,000	+
518	1:2,000–1:20,000	+
300	1:3,000–1:30,000	+
195	1:5,000–1:50,000	+
96	1:10,000–1:100,000	+
52	1:20,000–1:200,000	+
30	1:30,000–1:300,000	+
9	1:100,000–1:1,000,000	+
5	1:200,000–1:2,000,000	−
3	1:300,000–1:3,000,000	−

[a] BKG, background microflora.
[b] Confirmed by CR binding (YEP$^+$ cells appeared as red pinpoint colonies on CR-BHO) and PCR on day 6. +, present; −, absent.

agar did not prove to be effective in isolating clones that were eventually shown to be YEP$^+$ strains, likely due to the similar colonial morphology of other organisms present in the samples (11). Additionally, the antibiotic content of CIN agar may diminish the viability of YEP$^+$ strains. MAC agar gave consistently higher numbers of presumptives which were eventually shown to be YEP$^+$ strains. Ten percent of presumptive *Y. enterocolitica* from MAC agar was found to comprise YEP$^+$ strains.

Enrichment and isolation of presumptive YEP$^+$ strains were followed by identification by PCR assay and testing of plasmid-associated virulence phenotypes. The CR$^+$ clones were confirmed by multiplex PCR using primer pairs for the *ail* and *virF* genes which were confirmed to amplify a 170-bp product from the chromosome and a 591-bp product from the virulence plasmid, respectively (5) (data not shown). The specific presence of the *ail* gene in YEP$^+$ strains differentiates them from plasmid-bearing virulent *Y. pseudotuberculosis* strains lacking this gene (20). The presence of the *virF* gene demonstrates the presence of the virulence plasmid which confers plasmid-associated phenotypes.

From sample enrichment through confirmation, this method can be completed in 6 days and can recover YEP$^+$ strains in ground pork spiked with as low as 9 CFU/g (Table 1). This technique has been successfully applied in the recovery of different YEP$^+$ strains of five serotypes from artificially contaminated ground pork (Table 2). The procedure was tested on unspiked pork samples, including 20 ground pork samples, 10 head meat samples, and 30 tongue samples. Only tongue was found to contain YEP$^+$ strains (Table 3). A standard cold enrichment technique (12, 21) at 4°C for 4 weeks failed to isolate YEP$^+$ strains from any of these pork samples with which MAC and CIN agars were used as the selective media (data not presented). The successful isolation of YEP$^+$ strains from naturally contaminated tongue verified the effectiveness of this method. Of the 30 tongues analyzed, 11 were positive (~37%) by both CR binding and Lcr. PCR analysis confirmed the presence of a 170-bp product from the chromosomal and a 591-bp product from the virulence plasmid (5) (data not shown). All isolates from tongue were serotype O:3 and designated SB serotype (O:3). Recent reports have confirmed the emergence of *Y. enterocolitica* serotype O:3 as the major cause of yersiniosis in the United States (16, 17, 19).

TABLE 2. Isolation and confirmation of YEP$^+$ strains from artificially contaminated ground pork

Serotype	No. of strains tested	YEP$^+$ strain confirmation[a]
O:3	5	+
O:8	5	+
O:TACOMA	4	+
O:5, O:27	4	+
O:13	3	+

[a] See Table 1, footnote *b*.

TABLE 3. Recovery and confirmation of YEP$^+$ strains from pork samples

Type of pork sample	No. of samples	
	Tested	Having virulent YEP$^+$ strains
Ground meat	20	0
Head meat	10	0
Tongue	30	11

YEP$^+$ strains recovered from both artificially contaminated ground pork and naturally contaminated tongues were found to express plasmid-associated virulence characteristics, including colonial morphology (appearance of small colony size of 1.13 mm), CV binding (appearance of dark violet colony), Lcr in low-calcium medium (appearance of pinpoint colony size of 0.36 mm), CR uptake (appearance of red pinpoint colony size of 0.36 mm), HP by LPA test, and AA. The mouse virulence test of YEP$^+$ strain isolates from spiked ground pork and naturally contaminated tongue was positive for all samples. These results showed that the organism retained the virulence plasmid-associated phenotypic characteristics and pathogenicity after isolation from food by this procedure. Thus, the YEP$^+$ virulent strains were identified by both the presence of specific virulence genes and plasmid-associated phenotypic expression.

In conclusion, this recovery method uses a simple and efficient procedure that allows the recovery of various YEP$^+$ serotypes from pork samples. This procedure can be completed in 6 days, making it a practical alternative to many other enrichment methods which require significantly more time for completion.

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