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Enrichment Procedures and Plating Media for Isolation of *Yersinia enterocolitica*†

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ABSTRACT

A shortened enrichment procedure (25°C for 24 h) was compared with cold enrichment procedures (4°C for 1 to 3 weeks) and direct plating for isolation of *Yersinia enterocolitica* from commercial ground meat samples. The combined data of all recovery procedures showed that this organism was isolated from 34% of the ground beef samples. The highest isolation rate was 32% for the 4°C/3-week enrichment, followed by 28% for the 4°C/2-week enrichment, 26% for the 25°C/24-h enrichment, 22% for the 4°C/1-week enrichment, and 10% for direct plating. No significant differences ($P > 0.05$) in isolation rate occurred between the 4°C/3-week, 4°C/2-week, 25°C/24-h, and 4°C/1-week enrichments. The combined data of all recovery procedures showed that *Y. enterocolitica* was isolated from 64% of ground pork samples. The highest isolation rate was 48% for the 4°C/3-week enrichment, followed by 40% for the 25°C/24-h enrichment, 34% for the 4°C/2-week enrichment, 24% for the 4°C/1-week enrichment, and 24% for direct plating. No significant differences ($P > 0.05$) in isolation rate occurred between the 4°C/3-week, 25°C/24-h, and 4°C/2-week enrichments. During the plating phase of the experiment, the efficiency of a dye-containing, *Yersinia*-selective medium (KV202) was compared with that of a commercially available cefsulodin-irgasan-novobiocin medium. Recovery rates were similar for both media. However, KV202 agar differentiated *Y. enterocolitica* from such contaminating bacteria as *Enterobacter, Serratia,* and *Salmonella* by colony morphologic characteristics and color.

Yersinia enterocolitica is a zoonotic pathogen affecting both humans and animals. Swine has been implicated as the principal reservoir of human pathogenic *Y. enterocolitica (6).* Although *Y. enterocolitica* usually does not cause large outbreaks compared with other pathogens, this organism can grow at refrigerated temperatures because of its psychrotrophic nature. It recently has become recognized as an important emerging foodborne pathogen and has been isolated from a variety of foods, including pork, beef, lamb, raw milk, oyster, tofu, and fish *(7, 10, 12).* This organism has frequently been isolated from ground pork and other ground meats *(17).* Isolation of *Yersinia* from foods is not always easy because of the small number of *Yersinia* present and the great variety of background microflora in foods *(5).* Several methods have been proposed to isolate *Y. enterocolitica* from food and environmental specimens. Most methods involve enrichment of samples followed by plating onto selective media and confirmation of typical colonies. Because *Y. enterocolitica* can grow at 0 to 5°C, cold enrichment (at 4°C for 1 to 3 weeks) has been widely used *(3).* Although several kinds of selective media have been used as plating media for isolation of *Y. enterocolitica (2, 8),* cefsulodin-irgasan-novobiocin (CIN) agar is the most commonly used *(4).* However, some contaminating bacteria, such as *Citrobacter, Morganella, Pseudomonas,* and *Serratia,* can grow on CIN agar plates, making it difficult to confirm the presence of *Y. enterocolitica (11).*

Vichienroj and Fung *(16)* developed a new dye-containing, *Yersinia*-selective medium called KV202 agar. Several experiments have shown that KV202 agar is more selective than commonly used media and can differentiate between the growth of background microflora and *Y. enterocolitica.* However, cold enrichment requires a long incubation time, so shortening the enrichment time would make recovery of *Y. enterocolitica* more efficient.

The objectives of this study were to compare a shortened enrichment procedure with cold enrichment procedures and a direct plating method for recovery of *Y. enterocolitica* and to evaluate the efficiency of KV202 and CIN plating media for isolation and differentiation of *Y. enterocolitica* from commercial ground meat samples.

MATERIALS AND METHODS

Enrichment medium. Phosphate-buffered saline supplemented with 1% sorbitol and 0.15% bile salt (pH 7.6) *(1)* was used to enrich *Y. enterocolitica* from ground beef and ground pork samples.

Selective plating media. CIN agar (Oxoid, Basingstoke, UK) and KV202 agar (Bacto-peptone, 20.0 g; yeast extract, 2.0 g; dulcitol, 20.0 g; pyruvic acid, 2.0 g; sodium chloride, 1.0 g; magnesium sulfate, 0.01 g; sodium deoxycholate, 0.7 g; ferrous sulfate, 0.1 g; esculin, 1.0 g; brilliant yellow, 0.5 g; agar, 15.0 g; distilled water, 1,000 ml; cefsulodin, 15.0 mg; irgasan, 4.0 mg; and novobiocin 2.5 mg) *(16)* were used as plating agars for isolation of *Y. enterocolitica* from commercial ground beef and ground pork samples.

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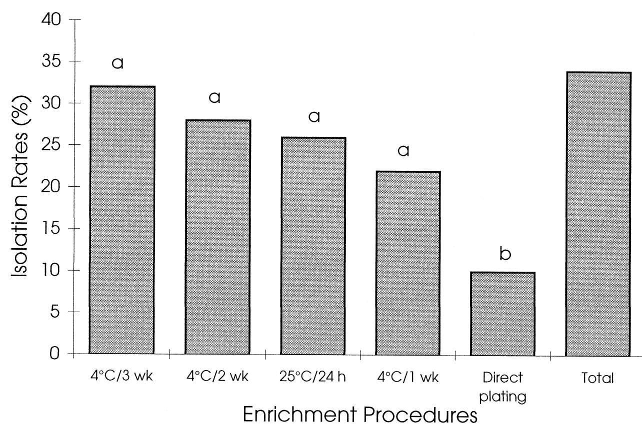

FIGURE 1. Yersinia enterocolitica *isolation rates from 50 ground beef samples by five recovery procedures. Bars with different letters are significantly different (*P *< 0.05).*

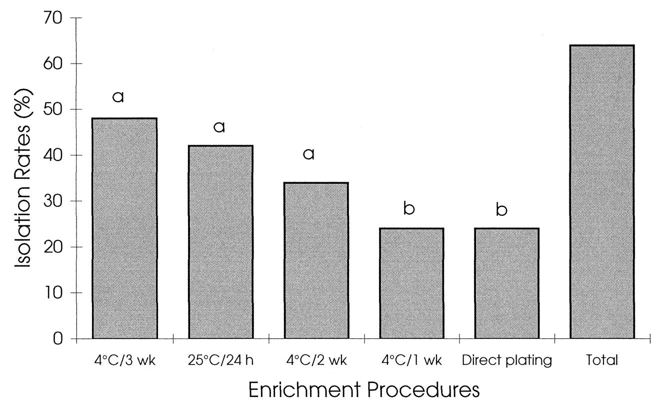

FIGURE 2. Yersinia enterocolitica *isolation rates from 50 ground pork samples by five recovery procedures. Bars with different letters are significantly different (*P *< 0.05).*

Collection of samples. Fifty ground beef and 50 ground pork samples (approximately 400 g each) were used to detect the presence of *Y. enterocolitica.* The samples were collected randomly from three local stores, held at −20°C, and analyzed within 1 week. All samples were held frozen before detection of *Y. enterocolitica.*

Preparation of samples and enrichment procedures. Two subsamples (25 g each) from each ground meat sample were aseptically placed into separate sterile stomach bags (Spiral Biotech, Inc., Bethesda, Md.) and homogenized in 225 ml of the enrichment broth for 2 min with use of a stomacher (model 400, Seward Medical, London, UK). One homogenized subsample was enriched using the shortened enrichment procedure (25°C for 24 h), and the other subsample was enriched using one of the cold enrichment procedures (4°C for 1 to 3 weeks).

Alkali treatment, plating, and confirmation of isolates. Enriched broth (0.5 ml) from the four enrichment procedures was placed in 4.5 ml of 0.5% KOH in 0.5% NaCl solution for 15 s, and then 0.05 ml of the treated broth was spread onto CIN and KV202 agar plates. For direct plating, 0.05 ml of homogenized sample was directly spread onto CIN and KV202 agar plates without enrichment or alkali treatment. For the 25°C enrichment, plating was performed after 24 h of incubation; for the 4°C enrichment, plating was performed weekly for 3 weeks with subcultures from the enrichment broth. Plates were incubated at 32°C for 24 h before observation of colony development. Suspected *Y. enterocolitica* colonies on agar plates were differentiated and identified by using triple sugar iron (TSI; Difco Laboratories, Detroit, Mich.) and an API-20E system (BioMerieux Vitek, Inc., Hazelwood, Mo.).

Statistical analysis. Differences in isolation rate between direct plating and the 25°C/24-h, 4°C/1-week, 4°C/2-week, and 4°C/3-week enrichments from 50 ground beef and 50 ground pork samples were examined by *t* test *(13).* Significance was defined as the 95% confidence limit ($P = 0.05$).

RESULTS AND DISCUSSION

Y. enterocolitica isolation rates of the five recovery procedures in 50 ground beef samples are presented in Figure 1. The highest isolation rate was 32% (16 of 50) for the 4°C/3-week enrichment, followed by 28% (14 of 50) for the 4°C/2-week enrichment, 26% (13 of 50) for the 25°C/24-h enrichment, 22% (11 of 50) for the 4°C/1-week enrichment, and 10% (5 of 50) for direct plating. No significant differences ($P > 0.05$) in isolation rate occurred between the 4°C/3-week, 4°C/2-week, 25°C/24-h, and 4°C/1-week enrichments. The combined isolation rates for all recovery procedures showed that *Y. enterocolitica* was isolated from a total of 34% of ground beef samples (17 of 50). Of all 17 positive samples, 13 were shown to be positive by both cold enrichment (4°C/1 to 3 weeks) and the shortened enrichment procedure (25°C for 24 h), 1 was shown to be positive only after enrichment at 25°C for 24 h, and 3 were shown to be positive only after cold enrichment (4°C for 1 to 3 weeks).

Isolation rates from the ground pork samples are presented in Figure 2. In the ground pork samples, the highest isolation rate was 48% (24 of 50) for the 4°C/3-week enrichment, followed by 42% (21 of 50) for the 25°C/24-h enrichment, 34% (17 of 50) for the 4°C/2-week enrichment, 24% (12 of 50) for the 4°C/1-week enrichment, and 24% (12 of 50) for direct plating. No significant differences ($P > 0.05$) in isolation rate were found between the 4°C/3-week, 25°C/24-h, and 4°C/2-week enrichments. The isolation rate for the 25°C/24-h enrichment was significantly higher ($P < 0.05$) than that for the 4°C/1-week enrichment and direct plating. The combined data of all recovery procedures showed that *Y. enterocolitica* was isolated from a total of 64% (32 of 50) of ground pork samples. Of all 32 positive samples, 25 samples were shown to be positive by both cold enrichment (4°C for 1 to 3 weeks) and the shortened enrichment procedure (25°C for 24 h), three were shown to be positive only by the 25°C/24-h enrichment, and four were shown to be positive only by cold enrichment (4°C for 1 to 3 weeks).

Both CIN and KV202 agar were used in the plating step. A sample was considered positive when *Y. enterocolitica* was isolated on either CIN or KV202 agar plates. In most cases, *Y. enterocolitica* was recovered from both agar plates.

The efficiencies of the different enrichment procedures are compared in Figures 1 and 2. In this study, the 4°C/3-week enrichment resulted in recovery of 94 and 75% of positive samples in ground beef and ground pork, respec-

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TABLE 1. *Isolation and identification of* Y. enterocolitica *from 50 ground beef samples by five recovery procedures*

Reaction	CIN	KV202
No. plates showing *Y. enterocolitica* suspect colonies	178/250 (71.3)[a]	109/250 (43.7)[a]
No. suspect colonies showing typical reaction on TSI medium	101/250 (40.4)[a]	79/250 (31.6)[a]
No. suspect colonies identified as *Y. enterocolitica*	57/250 (22.8)[a]	51/250 (20.4)[a]
No. false-positive suspect colonies	121/250 (48.4)[a]	58/250 (23.3)[a]
No. samples with *Y. enterocolitica*	17/50 (34.0)[b]	16/50 (32.0)[b]

[a] No. positive/no. agar plates examined (%).
[b] No. positive/no. meat samples (%).

tively. The 4°C/2-week enrichment resulted in recovery of 82 and 53% of positive samples in ground beef and ground pork, respectively. The 25°C/24-h enrichment resulted in recovery of 76 and 66% of positive samples in ground beef and ground pork, respectively. These results indicate that the 25°C/24-h enrichment was as effective as 2 to 3 weeks of enrichment at 4°C in recovery of *Y. enterocolitica* from ground beef and ground pork samples. A similar result was reported by Doyle and Hugdahl *(5),* who found that 1 to 3 days of enrichment at 25°C was as effective as 14 to 21 days at 4°C in recovery of *Y. enterocolitica* in meats.

Cold enrichment of food samples may result in growth of other psychrotrophic bacteria, such as *Pseudomonas fluoresens* and *Serratia liquefaciens (14).* These competitor bacteria can grow on CIN agar plates and are difficult to differentiate from *Y. enterocolitica.* The direct plating procedure resulted in recovery of only 29 and 38% of positive samples in ground beef and ground pork, respectively, when this common plating medium was used. This procedure is far less effective than the cold enrichment and 25°C/24-h enrichment procedures. Although no enrichment procedure recovered *Y. enterocolitica* from all positive samples, enrichment at 25°C for 24 h substantially reduced the amount of recovery time and could serve as a useful alternative to cold enrichment.

The efficiencies of CIN and KV202 agar in recovery of *Y. enterocolitica* from 50 ground beef samples using the five recovery procedures are compared in Table 1. For the 50 ground beef samples, 250 CIN and 250 KV202 agar plates were used in the plating step. A total of 178 and 109 plates showed suspected *Y. enterocolitica* colonies on CIN and KV202 agar, respectively. After inoculation of these colonies into TSI medium, 101 and 79 isolates from CIN and KV202 agar, respectively, exhibited typical *Y. enterocolitica* reactions (acid butt and acid slant, without gas, no H_2S production). Confirmation of these TSI-positive isolates with use of the API-20E system showed that 57 isolates from CIN agar and 51 isolates from KV202 agar were *Y. enterocolitica.* These isolates represented 16 positive samples from both CIN and KV202 agar and one positive sample from CIN agar only.

The efficiencies of CIN and KV202 agar in the recovery of *Y. enterocolitica* from 50 ground pork samples using five recovery procedures are compared in Table 2. For the 50 ground pork samples, a total of 187 and 128 plates showed suspected *Y. enterocolitica* colonies on CIN and KV202 agar, respectively. After inoculation of these colonies into TSI medium, 112 and 87 isolates from CIN and KV202 agar, respectively, exhibited typical *Y. enterocolitica* reactions (acid butt and acid slant, without gas, no H_2S production). Confirmation of these TSI-positive isolates with use of the API-20E system showed that 64 isolates from CIN and 60 isolates from KV202 agar were *Y. enterocolitica.* These isolates represented 28 positive samples from both CIN and KV202 agar; three positive samples were from CIN agar only, and one was from KV202 agar only.

Although CIN agar yielded a slightly higher ($P > 0.05$) recovery rate of *Y. enterocolitica* than KV202 agar from ground meat samples, CIN agar indicated many more false-positive suspect colonies than KV202 agar. The false-positive colony ratios were 48.4% on CIN agar and 23.3% on KV202 agar for the 50 ground beef samples (Table 1). For the 50 ground pork samples, 49.2% of the colonies identified as suspect on CIN agar and 27.2% of those identified on KV202 agar were false-positive colonies. Thus, more TSI medium and API-20E test strips were used for CIN agar plates, and more time was required for differentiation and identification of suspected colonies.

Use of KV202 agar resulted in fewer false-positive results and improved differentiation properties. Differentiating *Yersinia* from other competing bacteria colonies on CIN

TABLE 2. *Isolation and identification of* Y. enterocolitica *from 50 ground pork samples by five recovery procedures*

Reaction	CIN	KV202
No. plates showing *Y. enterocolitica* suspect colonies	187/250 (74.7)[a]	128/250 (51.0)[a]
No. suspect colonies showing typical reaction on TSI medium	112/250 (44.8)[a]	87/250 (34.8)[a]
No. suspect colonies identified as *Y. enterocolitica*	64/250 (25.6)[a]	60/250 (24.0)[a]
No. false-positive suspect colonies	123/250 (49.2)[a]	68/250 (27.2)[a]
No. samples with *Y. enterocolitica*	31/50 (62.0)[b]	29/50 (58.0)[b]

[a] No. positive/no. agar plates examined (%).
[b] No. positive/no. meat samples (%).

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agar is difficult, because *Yersinia, Serratia, Enterobacter,* and *Salmonella* produce similar red colonies on this medium *(16)*. Colonies of *Serratia liquefaciens, Citrobacter freundii,* and *Enterobacter agglomerans* cannot be differentiated from colonies of *Y. enterocolitica* on CIN agar *(9)*. KV202 agar can support the growth of common virulent *Y. enterocolitica* serotypes, such as O:3, O:8, and O:9 *(15)*, and also differentiate *Y. enterocolitica* from other bacteria. On KV202 agar plates, *Serratia* and *Enterobacter* produce yellow colonies with a black zone around the colonies after 24 h of incubation at 32°C; *Salmonella* produces yellow colonies with a red zone; and *Y. enterocolitica* produces light yellow colonies without a black zone after 24 h of incubation. Thus, use of KV202 agar plates facilitates differentiation of *Y. enterocolitica* from other organisms.

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