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New Enrichment Method for Isolation of Pathogenic *Yersinia enterocolitica* Serogroup O:3 from Pork

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A new enrichment medium for the recovery of pathogenic *Yersinia enterocolitica* serogroup O:3 from naturally infected meat products based on three selective agents, Irgasan, ticarcillin, and potassium chlorate (ITC), was compared with several other one- or two-step enrichments. *Y. enterocolitica* serogroup O:3 was recovered from 96.5% of 29 pork tongues, 24% of 50 ground pork samples, 16% of 25 masseter muscle samples, and 61% of tonsils. ITC was by far the most sensitive method for the recovery of *Y. enterocolitica* O:3, especially from ground meat and masseter muscles, while cold and two-step enrichments yielded better results for nonpathogenic strains. Plating of ITC enrichments onto SS-deoxycholate-calcium agar gave overall better results than plating onto cefsulodin-Irgasan-novobiocin agar for serogroup O:3.

The recovery of *Yersinia enterocolitica* from foodstuffs usually requires the use of enrichment techniques, because of the small number of pathogens and the large number of contaminating bacteria. Fukushima (8), however, succeeded in isolating small numbers of pathogenic *Yersinia* species from meat by direct plating after alkali treatment.

Several enrichment methods have been proposed for the isolation of *Y. enterocolitica* from meat and other foods. Cold enrichment for 2 or 3 weeks in buffered saline (11) or in a richer peptone-containing broth (19) has widely been used. A short 2- or 3-day enrichment in a modified Rappaport broth with carbenicillin was developed for the pathogenic strains of serogroups O:3 and O:9 (20). A selenite-malachite green-carbenicillin enrichment without added nutrients was recommended by Lee et al. (10), especially for the recovery of more fastidious strains from meat. Schiemann (15) described a two-step enrichment, suitable for the different types of *Y. enterocolitica*. Several other methods or modifications of existing methods have been proposed but are less widely used.

We developed a new enrichment broth derived from the modified Rappaport base, supplemented with Irgasan, ticarcillin, and potassium chlorate (ITC). This medium was evaluated for the recovery of pathogenic *Y. enterocolitica*, especially serogroup O:3, in comparison with other currently used enrichment methods. Naturally infected pork meat products were tested since healthy slaughter pigs are known to be carriers of pathogenic *Yersinia* species (1, 5, 7, 8, 12, 14, 16, 18, 21, 24) and constitute the only important reservoir for human infections in Belgium (17).

MATERIALS AND METHODS

In a preliminary study (unpublished data), the new enrichment broth proved to be superior to the previously described modified Rappaport broth (20), which was therefore not included in this study. The new medium was compared with different combinations of the following procedures: direct plating after alkali treatment, two-step enrichment by the method of Schiemann (15), modified selenite used as a preenrichment, and cold enrichment in buffered saline followed or not followed by alkali treatment.

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Enrichment techniques. The new enrichment broth, called ITC, was prepared with the following materials: tryptone, 10 g; yeast extract, 1 g; $MgCl_2 \cdot 6H_2O$, 60 g; NaCl, 5 g; $KClO_3$, 1 g; 0.2% malachite green solution, 5 ml; and distilled water to make 1,000 ml. After sterilization, ticarcillin and Irgasan DP 300, as the 2,4,4'-trichloro-2'-hydroxydiphenyl ether (CIBA-GEIGY, Basel, Switzerland), were added at a final concentration of 1 μg/ml each. The medium was dispensed in 10-ml amounts in test tubes (16 by 160 mm) for small inocula and subcultures and in 100-ml amounts in Erlenmeyer flasks for larger inocula.

Modified selenite medium (SEL) was prepared by the method of Lee et al. (10) with the low concentration of selenite.

Alkali treatment was performed by the method of Aulisio et al. (2) with a 0.25% KOH solution.

Two-step enrichment was that of Schiemann (15). The preenrichment medium (PEM) was slightly modified at the suggestion of Schiemann (personal communication) to consist of 0.05 M Na_2HPO_4 containing final concentrations of the following products: 0.1% NaCl; 0.1% KCl; 1.0% special peptone (Oxoid Ltd., London, England); 2.0% yeast extract. The pH was adjusted to 8.3, and after autoclaving, two filter-sterilized solutions were added to obtain the following final concentrations: 0.001% $MgSO_4 \cdot 7H_2O$ and 0.001% $CaCl_2$. The bile-oxalate-sorbose enrichment (BOS) was used as originally described by Schiemann (15).

Phosphate-buffered saline (PBS) (pH 7.6) was used for the conventional cold enrichment at 4°C.

Plating media. Two selective agars were used: cefsulodin-Irgasan-novobiocin agar (CIN) (Oxoid) (13) and modified SS-deoxycholate agar (20), containing 1% sodium deoxycholate and 0.1% $CaCl_2$ (SSDC) (yersinia-agar; E. Merck AG, Darmstadt, Federal Republic of Germany). These media were incubated at 30°C for 24 h. All enrichment media were subcultured onto the two plating media unless otherwise indicated. KOH-treated samples were only plated onto CIN, because SSDC proved to be unsuitable after alkali treatment.

Meat samples. Fifty samples of ground pork (or mixed pork-beef) were purchased from several retail shops. Twenty-nine pork tongues were similarly obtained.

Two batches of masseter muscles were removed from the

TABLE 1. Comparative isolation of pathogenic *Y. enterocolitica* O:3 and other nonpathogenic types from pork tongues (29 samples) by different methods

Enrichment protocol	No. of positive samples after being plated onto:			
	CIN		SSDC	
	O:3	Others	O:3	Others
Direct plating after KOH	17	7	ND[a]	ND
ITC, 2 or 3 days at 24°C	19	1	28	1
PEM + ITC	12	6	19	5
PEM + BOS	0	1	0	0
PBS (4°C) + ITC	6	5	10	4
PBS (4°C) + KOH	4	9	ND	ND

[a] ND, Not done.

pigs at slaughter and stored at −28°C. Masseter muscles are used for the preparation of ground pork to be sold as raw ground meat. Twenty-five samples from each batch were examined.

Fifty-four tonsil samples were collected from pigs at slaughter.

Inoculation and incubation procedures. (i) Pork tongues. The surface of the tongue was swabbed to prepare a turbid suspension in approximately 3 ml of PBS. This suspension was used as inoculum for the following procedures. (i) The inoculum was treated with KOH and directly plated onto CIN. (ii) One milliliter was transferred into 100 ml of ITC and incubated for 2 or 3 days at 24°C. (iii) One milliliter was transferred into 10 ml of ITC and incubated for 2 or 3 days at 24°C. (iv) One milliliter was transferred into 10 ml of PEM and incubated for 7 days at 4°C; 0.1 ml of this preenrichment was inoculated into 10 ml of BOS and incubated for 7 days at 24°C. A further 0.1 ml of PEM was also inoculated into 10 ml of ITC and incubated for 2 days at 24°C. (v) One milliliter was transferred into 10 ml of PBS and incubated for 2 weeks at 4°C. This cold enrichment was plated onto CIN agar after KOH treatment, and 0.1 ml was inoculated into ITC as a second-step enrichment.

(ii) Ground pork-beef. Ground pork-beef (5 g) was thoroughly mixed with 20 ml of PBS. The liquid part of this 20% suspension served as the inoculum for some media, while the meat itself was used as the inoculum in other instances, as follows. (i) The inoculum was treated with KOH and directly plated onto CIN. (ii) One milliliter of the suspension and 10 g of meat were each added to 100-ml volumes of ITC and incubated for 2 or 3 days at 24°C. (iii) Meat (10 g) was transferred into 100 ml of PEM and incubated for 7 days at 4°C, followed by subcultures into ITC and BOS as above. (iv) Meat (10 g) was transferred into 100 ml of SEL and incubated for 3 days at 24°C. SEL was also used as a preenrichment, and 0.1 ml was subcultured into ITC. (v) The suspension as such was kept for 2 weeks at 4°C as a cold enrichment and then treated with KOH and plated onto CIN.

(iii) Pork masseter muscles. Masseter muscles were processed as described above and inoculated as follows. (i) A 0.3-ml sample was mixed into 10 ml of ITC and incubated for 2 days at 24°C, followed by subculture onto SSDC only. (ii) Meat (5 g) was transferred into 20 ml of PEM and incubated for 7 days at 4°C. This was followed by subculture of 0.5 ml into 10 ml of BOS, incubation for 7 days at 24°C, and subculture onto CIN only.

(iv) Pork tonsils. A 20% pork tonsil suspension in PBS was prepared by homogenizing a sample of approximately 5 g in a stomacher for 1 min. A 1-ml sample of this suspension was transferred into 100 ml of ITC and incubated for 2 days at 24°C. The remainder was inoculated into 100 ml of SEL. After incubation for 2 days at 24°C, 0.1 ml was subcultured into 10 ml of ITC.

Identification and typing methods. All *Yersinia* isolates were identified to the species level, and all *Y. enterocolitica* isolates were biotyped and serotyped by previously described methods (4, 22). Isolates were divided into two categories: serogroup O:3 (biogroup 4) and serogroups O:9 and O:5,27 (biogroup 2) are the only recognized human pathogens in Belgium (6). The other species and types, particularly those positive for the esculin or pyrazinamidase test or both, were considered as nonpathogenic (23).

RESULTS

Pork tongues. *Y. enterocolitica* biogroup 4 serogroup O:3 was isolated from 28 of the 29 pork tongue samples (96.5%). Other, nonpathogenic strains, including many serogroups of esculin-positive biogroup 1 strains and related *Yersinia* spp., were found in 19 samples (65.5%).

The recovery of pathogenic and nonpathogenic strains on different media is presented in Table 1. More than 50% of O:3 strains were isolated by direct plating after KOH treatment. All the O:3 strains were recovered by the short ITC enrichment followed by subculture onto SSDC agar. There was a marked loss of those strains after preenrichment or cold enrichment.

Ground pork. From 50 samples of ground pork, 12 O:3 strains were isolated, an overall positivity rate of 24%. No other pathogenic serogroups were found. Forty samples (80%) were positive for nonpathogenic strains belonging to various other serogroups of *Y. enterocolitica* and related species. Table 2 shows the recovery rate according to the different methods used. Except for one strain obtained by direct plating, all the O:3 strains were isolated after enrichment in either ITC broth or SEL broth. The latter was followed or not followed by subculture into ITC and plating onto SSDC. Of the 12 strains, 10 were isolated by ITC enrichment and 7 were recovered only by this method, while 2 strains were found only by SEL enrichment or SEL followed by ITC. The isolation of nonpathogenic strains was markedly increased after preenrichment and cold enrichment. Subculture on SSDC was clearly more selective for serogroup O:3, while CIN medium yielded significantly more nonpathogenic strains.

Masseter muscles. For masseter muscles, two batches of 25 samples each were examined. ITC plated onto SSDC was

TABLE 2. Comparative isolation of pathogenic *Y. enterocolitica* O:3 and other, nonpathogenic types from ground pork (50 samples) by different methods

Enrichment protocol	No. of positive samples after being plated onto:			
	CIN		SSDC	
	O:3	Others	O:3	Others
Direct plating after KOH	1	6	ND[a]	ND
ITC light inoculum (1/100)	1	3	10	3
ITC heavy inoculum (10/100)	1	0	2	0
SEL	1	1	0	0
SEL + ITC	0	5	4	8
PEM + ITC	0	10	0	10
PEM + BOS	0	16	0	1
PBS (4°C) + KOH	0	20	ND	ND

[a] ND, Not done.

compared with PEM plus BOS plated onto CIN. The results are summarized in Table 3. In the first batch, 20 samples (80%) were positive for nonpathogenic strains and 11 samples contained two or more serogroups. Eleven samples were only positive after PEM plus BOS followed by plating onto CIN, while a single sample was only positive after ITC followed by SSDC. The second batch yielded O:3 strains in four samples (16%) which were recovered only by the combination of ITC and SSDC. No other strains were isolated.

Tonsils from slaughter pigs. Of the 54 tonsil samples examined, O:3 strains were isolated from 33 (61%) and other strains belonging to biogroup 1 were isolated from 7 (13%). ITC alone was compared with ITC after SEL, both plated onto CIN and SSDC. ITC alone was slightly but not significantly better than when preceded by SEL for serogroup O:3 strains, which were also slightly but not significantly better recovered on SSDC than on CIN (Table 4).

DISCUSSION

ITC enrichment broth is derived from the modified Rappaport broth, which has proven to be suitable for the recovery of *Y. enterocolitica* O:3 from various types of infected material (10, 15, 21, 24). Apart from the magnesium chloride-malachite green base, the selectivity of the new medium is mainly based on three agents: Irgasan, ticarcillin, and potassium chlorate.

Irgasan was demonstrated to be selective for *Yersinia* species by Schiemann (13) and is one of the main selective agents of CIN agar. Ticarcillin has the same activity as carbenicillin. Schiemann (15) has suggested deleting carbenicillin from the modified Rappaport broth since it is inhibitory for some *Yersinia* strains. This does not apply to serogroup O:3, and deletion of either carbenicillin or ticarcillin results in a decrease of selectivity. Potassium chlorate was first used by Bercovier et al. (3) as a selective agent in a solid plating medium incubated anaerobically. The inhibitory activity of chlorate is based on the presence in most members of the family *Enterobacteriaceae* of a A-nitratase capable of splitting chlorate, which then becomes toxic for those strains. *Yersinia* species have a B-nitratase without activity on chlorate. Since A-nitratase is only inducible under reduced oxygen pressure, it is mandatory to achieve a small surface/volume ratio of the medium. Tubes for small volumes and Erlenmeyer flasks for larger amounts are satisfactory in this respect.

When ITC enrichment was compared with direct plating or other enrichment methods in our study, it always yielded the highest recovery rate for *Y. enterocolitica* O:3. Direct plating after KOH treatment was only suitable for strongly positive material like pork tongues, while our isolation rate from ground meat was very low: 1 strain versus 12 after enrichment. Fukushima (8) achieved better results by direct plating than by enrichment, only one O:3 strain being recovered after enrichment versus five recovered by direct plating. However, it must be noted that only cold enrichment was used by this investigator.

TABLE 3. Comparative isolation of pathogenic *Y. enterocolitica* O:3 and other nonpathogenic types from masseter muscles (50 samples) by two enrichment methods

Enrichment protocol	No. of positive samples after being plated onto:			
	CIN		SSDC	
	O:3	Others	O:3	Others
Batch 1 (25 samples)				
ITC	ND[a]	ND	0	7
PEM + BOS	0	19	ND	ND
Batch 2 (25 samples)				
ITC	ND	ND	4	0
PEM + BOS	0	0	ND	ND

[a] ND, Not done.

TABLE 4. Isolation of pathogenic *Y. enterocolitica* O:3 and other nonpathogenic types from tonsils of slaughter pigs (54 samples) by ITC enrichment with and without SEL preenrichment

Enrichment protocol	No. of positive samples after being plated onto:			
	CIN		SSDC	
	O:3	Others	O:3	Others
ITC	26	1	28	3
SEL + ITC	19	4	23	4

Although SEL enrichment allowed isolation of a few pathogenic strains from ground meat, we cannot compare our results with those described by Lee et al. (10) since the inoculum size recommended by these researchers was smaller and our purpose was rather to use it as a preenrichment.

A two-step enrichment on BOS broth after a preenrichment was recommended by Schiemann (15) as a multivalent method that would support growth of all *Yersinia* strains, including the most fastidious. A great variety of strains were indeed recovered by this technique from ground meat, but the recovery of serogroup O:3 strains was disappointing. Similarly, cold enrichment in PBS was suitable for nonpathogenic strains but results were poor for serogroup O:3. This was already reported by other workers (10, 21, 24).

Better results were achieved for ground meat in ITC medium by using an inoculum/medium ratio of 1/100 rather than 1/10, although the inoculum size was smaller in the former case. A relatively heavy inoculum usually results in an overgrowth of contaminating bacteria. Lee et al. (10) already made the same observation for the SEL enrichment. However, we did not apply KOH treatment when subculturing these media, and this treatment might have improved the recovery of *Yersinia* species from heavily inoculated enrichments.

CIN agar is generally accepted as the most valuable plating medium for the isolation of the different *Yersinia* species and biogroups and serogroups (15). However, in combination with ITC enrichment, recovery of serogroup O:3 was usually better on SSDC in our study. From a total of 524 subcultures of different liquid enrichments on the two plating media, 85 were positive for *Yersinia* serogroup O:3 on CIN and 125 were positive on SSDC. Two *Yersinia* serogroup O:3 strains were recovered only on CIN, and 42 were recovered only on SSDC ($P < 0.001$). The addition of calcium to the original formulation of SSDC markedly enhanced the growth of O:3 strains, and their colonies at 24 h of incubation were larger than on CIN. Moreover, the predictive value of the colony aspect for serogroup O:3 with a stereomicroscope was better on SSDC (20), whereas on CIN agar no typical aspect could be detected for those strains. The new medium, VYE, described by Fukushima (9) could perhaps provide a better approach to this problem. Furthermore, Irgasan is an important component of ITC and is also a main selective agent of CIN. It might be less

effective to select successively with the same agent in the enrichment broth and the plating agar. Conversely, SSDC is less suitable for other pathogenic and for nonpathogenic *Yersinia* strains.

It is obvious from our and other studies that the behavior of *Yersinia* species on different selective and enrichment media may vary considerably according to the biogroups-serogroups. The main drawback of ITC broth might be that its efficacy is only demonstrated for *Y. enterocolitica* serogroup O:3. However, no other pathogenic strains were isolated from the various materials used in this survey by either method. There is limited evidence that the pathogenic serogroup O:9 can be satisfactorily enriched by ITC from human stool cultures (unpublished data). Other pathogenic strains might grow less well on this medium, but extended trials on naturally infected material are necessary to correctly evaluate the suitability of this method for such strains.

The aim of many previous investigations was to find a multivalent isolation method suitable for all *Yersinia* spp. or all pathogenic *Y. enterocolitica* simultaneously. Although some of these methods theoretically allow recovery of several types of *Yersinia* species, no one method seems to give an optimal recovery for all the pathogenic strains. In this study, most pathogenic O:3 strains would have been missed by the use of conventional multivalent methods, particularly in material like ground meat, containing low numbers of such organisms, but heavily contaminated by other bacteria. Since serogroup O:3 is one of the most important in humans and prevails in many countries worldwide, enrichment in ITC would be advisable, at least in addition to other methods.

LITERATURE CITED

1. **Asakawa, Y., S. Akahane, K. Shiozawa, and T. Homma.** 1979. Investigations of source and route of *Yersinia enterocolitica* infection. Contrib. Microbiol. Immunol. **5**:115–121.
2. **Aulisio, C. C. G., I. J. Mehlman, and A. C. Sanders.** 1980. Alkali method for rapid recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from foods. Appl. Environ. Microbiol. **39**:135–140.
3. **Bercovier, H., J. Brault, S. Cohen, R. Melis, T. Lambert, and H. H. Mollaret.** 1984. A new isolation medium for the recovery of *Yersinia enterocolitica* from environmental sources. Curr. Microbiol. **10**:121–124.
4. **Bercovier, H., D. J. Brenner, J. Ursing, A. G. Steigerwalt, G. R. Fanning, J. M. Alonso, G. P. Carter, and H. H. Mollaret.** 1980. Characterization of *Yersinia enterocolitica sensu stricto*. Curr. Microbiol. **4**:201–206.
5. **Christensen, S. G.** 1987. The *Yersinia enterocolitica* situation in Denmark. Contrib. Microbiol. Immunol. **9**:93–97.
6. **De Groote, G., J. Vandepitte, and G. Wauters.** 1982. Surveillance of human *Yersinia enterocolitica* infections in Belgium: 1963-1978. J. Infect. **4**:189–197.
7. **Doyle, M. P., and M. B. Hugdahl.** 1983. Improved procedure for recovery of *Yersinia enterocolitica* from meats. Appl. Environ. Microbiol. **45**:127–135.
8. **Fukushima, H.** 1985. Direct isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from meat. Appl. Environ. Microbiol. **50**:710–712.
9. **Fukushima, H.** 1987. New selective agar medium for isolation of virulent *Yersinia enterocolitica*. J. Clin. Microbiol. **25**:1068–1073.
10. **Lee, W. H., M. E. Harris, D. McClain, R. E. Smith, and R. W. Johnston.** 1980. Two modified selenite media for the recovery of *Yersinia enterocolitica* from meats. Appl. Environ. Microbiol. **39**:205–209.
11. **Paterson, J. S., and R. Cook.** 1963. A method for the recovery of *Pasteurella pseudotuberculosis* from faeces. J. Pathol. Bacteriol. **85**:241–242.
12. **Pedersen, K. B.** 1979. Occurrence of *Yersinia enterocolitica* in the throat of swine. Contrib. Microbiol. Immunol. **5**:253–256.
13. **Schiemann, D. A.** 1979. Synthesis of a selective agar medium for *Yersinia enterocolitica*. Can. J. Microbiol. **25**:1298–1304.
14. **Schiemann, D. A.** 1980. Isolation of toxigenic *Yersinia enterocolitica* from retail pork products. J. Food Prot. **43**:360–365.
15. **Schiemann, D. A.** 1982. Development of a two-step enrichment procedure for recovery of *Yersinia enterocolitica* from food. Appl. Environ. Microbiol. **43**:14–27.
16. **Stengel, G.** 1984. *Yersinia enterocolitica*; Vorkommen und Bedeutung in Lebensmitteln, p. 102–108. *In* Proceedings of the 25th Deutsche Veterinaermedizinische Gesellschaft Conference, Garmisch-Partenkirchen, Federal Republic of Germany. Deutsche Veterinaermedizinische Gesellschaft E. V., Giessens-Lhan, Federal Republic of Germany.
17. **Tauxe, R. V., J. Vandepitte, G. Wauters, S. M. Martin, V. Goossens, P. De Mol, R. Van Noyen, and G. Thiers.** 1987. *Yersinia enterocolitica* infections and pork: the missing link. Lancet **i**:1129–1132.
18. **Toma, S., and V. R. Deidrick.** 1975. Isolation of *Yersinia enterocolitica* from swine. J. Clin. Microbiol. **2**:478–481.
19. **Van Pee, W., and J. Stragier.** 1979. Evaluation of some cold enrichment and isolation media for the recovery of *Yersinia enterocolitica*. Antonie van Leeuwenhoek J. Microbiol. **45**:465–477.
20. **Wauters, G.** 1973. Improved methods for the isolation and the recognition of *Yersinia enterocolitica*. Contrib. Microbiol. Immunol. **2**:68–70.
21. **Wauters, G.** 1979. Carriage of *Yersinia enterocolitica* serotype 3 by pigs as a source of human infection. Contrib. Microbiol. Immunol. **5**:249–252.
22. **Wauters, G.** 1981. Antigens of *Yersinia enterocolitica*, p. 41–53. *In* E. Bottone (ed.), *Yersinia enterocolitica*. CRC Press, Inc., Boca Raton, Fla.
23. **Wauters, G., K. Kandolo, and M. Janssens.** 1987. Revised biogrouping scheme of *Yersinia enterocolitica*. Contrib. Microbiol. Immunol. **9**:14–21.
24. **Weber, A., and C. Lembke.** 1981. Vergleichende Anwendung von zwei Anreichnungsverfharen und funf Selektivnährboden zur Isolierung von *Yersinia enterocolitica* aus Tonsillen von Schlachtschweinen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **250**:72–77.