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Low Occurrence of Pathogenic *Yersinia enterocolitica* in Clinical, Food, and Environmental Samples: a Methodological Problem

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INTRODUCTION

Yersinia enterocolitica, a gram-negative, oxidase-negative, and facultatively anaerobic species, is highly heterogeneous and can be divided into several bioserotypes, only a few of which are known to associate with human disease (18, 121). Most *Y. enterocolitica* strains associated with human yersiniosis belong to bioserotypes 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3. All fully virulent *Yersinia* strains carry an approximately 70-kb plasmid termed pYV (plasmid for *Yersinia* virulence) (116), which is essential for the bacterium to survive and multiply in lymphoid tissues (21, 26).

Although *Y. enterocolitica* is a ubiquitous microorganism, the majority of isolates recovered from asymptomatic carriers, food, and environmental samples are nonpathogenic. Nevertheless, it is important to determine the pathogenic significance of isolates (28, 76). This can be done with several phenotypic tests, but these are time-consuming and are not always reliable (83). PCR and DNA colony hybridization assays have been used to verify the pathogenicity of *Y. enterocolitica* isolates rapidly and with high specificity (13, 77, 163). These methods are based on specific segments, such as *yadA* and *virF* genes, of the virulence plasmid.

The epidemiology of *Y. enterocolitica* infections is complex and poorly understood. Most cases of yersiniosis occur sporadically without an apparent source (19, 76, 113, 139). *Y. enterocolitica* is thought to be a significant food-borne pathogen, although pathogenic isolates have seldom been isolated from foods, except from edible pig offal (28, 47, 48). In case-control studies, a correlation has been demonstrated between the consumption of raw or undercooked pork and yersiniosis (114, 127, 141). Genotypes of *Y. enterocolitica* strains found in pigs and pork are indistinguishable from strains found in humans, further supporting the association between yersiniosis and consumption of pork (44).

Difficulties associated with the isolation of pathogenic *Y. enterocolitica* stem from the small number of pathogenic strains in the samples and the large number of organisms in the background flora, especially in food and environmental samples. Direct isolation, even on selective media, is seldom successful, and time-consuming enrichment steps are needed. No single procedure is currently available which will recover all pathogenic serotypes (27). The low rates of isolation of pathogenic *Y. enterocolitica* in natural samples may be due to the limited sensitivity of culture methods (109). Using DNA-based methods, including PCR and DNA colony hybridization, this pathogen can be detected more rapidly and with greater sensitivity (62, 70, 123).

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CULTURE METHODS

The source of *Y. enterocolitica* can markedly affect the methods of isolation. It is generally easier to find pathogenic isolates

TABLE 1. Methods of isolation of *Y. enterocolitica* most commonly used for clinical, food, and environmental samples

Preenrichment[a]	Selective enrichment[a]	Postenrichment	Selective agar plate	Serotype(s) recovered	Reference(s)
PSB, 4°C, 3–4 wk			MAC, 25°C, 48 h, CIN, 30°C, 24 h	All	99, 112a
PSB, 10°C, 10 days		KOH	CIN and MAC, 22–26°C, 48 h	All	39
PBS, 4°C, 21 days			CIN, 30°C, 24–48 h	All	4
PBS/PSB, 25°C, 1–3 days		KOH	MAC, 25°C, 48 h; CIN, 30°C, 24 h	All	35, 68
	SEL, 22°C, 3 days		MAC, 25°C, 48 h	O:3, O:8	91
PSB, 4°C, 8 days	MRB, 22°C, 4 days		CIN, 30°C, 24 h	O:3, O:9	112a, 130
YER, 4°C, 9 days	BOS, 22°C, 5 days		CIN, 30°C, 24 h	O:3, O:8	130
TSB, 22°C, 1 day	BOS, 22°C, 7 days		CIN, 30°C, 24 h	O:3, O:8	131
	ITC, 25°C, 2 days		SSDC, 30°C, 24 h	O:3	68, 158

[a] SEL, selenite broth; YER, yeast extract-rosebengal broth.

in clinical specimens from infected individuals than in asymptomatic carriers, foods, or environmental samples. In patients with acute gastroenteritis or organ abscesses, pathogenic *Y. enterocolitica* is often the dominant bacteria and can readily be isolated by direct plating on conventional enteric media (4). However, because of the small number of pathogenic strains of *Yersinia* in asymptomatic carriers and the large number of organisms in the background flora in food and environmental samples, direct isolation, even on selective media, is seldom successful. To increase the number of *Yersinia* strains in these samples, enrichment in liquid media prior to isolation on solid media is required (27). Several different methods available for isolation of *Y. enterocolitica* are presented in Table 1.

Cold Enrichment

The psychrotrophic nature of *Y. enterocolitica* is unusual among other *Enterobacteriaceae*; consequently, enrichment in different solutions at 4°C for prolonged periods has been used for isolation of *Yersinia* spp. Cold enrichment in phosphate-buffered saline (PBS) or in phosphate-buffered saline with sorbitol and bile salts (PSB) has been widely used for clinical, food, and environmental samples (29, 53, 92, 95, 108, 137). Cold enrichment is effective for fecal samples in some studies (63, 82); however, major disadvantages are the presence of nonpathogenic *Y. enterocolitica* and other psychrotrophic bacteria, which also multiply during enrichment, and the long incubation period, typically 21 days. By treating cold enrichments with potassium hydroxide (KOH), the background flora can sometimes be reduced, making selection of *Yersinia* colonies less laborious (132).

Selective Enrichment

Several selective media for isolation of *Y. enterocolitica* at higher temperatures have been developed with different antimicrobial agents used as selective supplements. Wauters (156) formulated a modified Rappaport broth (MRB), containing magnesium chloride, malachite green, and carbenicillin, in which the sample is incubated at 25°C for 2 to 4 days. Wauters et al. (158) subsequently developed an enrichment broth derived from the modified Rappaport base, supplemented with irgasan, ticarcillin, and potassium chlorate (ITC). Both media have been useful in recovery of strains of bioserotype 4/O:3 (29, 72, 85, 108, 158). Schiemann (130) developed a bile-oxalate-sorbose (BOS) medium for the isolation of *Y. enterocolitica,* particularly for bioserotype 1B/O:8 strains. Two lesser known enrichment procedures based on tryptic soy broth (TSB) have been proposed later. Landgraf et al. (87) used TSB with polymyxin and novobiocin (TSPN) and incubation at 18°C for 3 days for isolation of *Y. enterocolitica* in milk. Toora et al. (145) designed a two-step procedure for isolation of *Y. enterocolitica* from ready-to-eat foods and pork by using modified TSB containing yeast extract, bile salts, and irgasan.

Selective Agar Plates

Many different selective agar plating media are available for isolation of *Y. enterocolitica* from clinical, food, and environmental samples. Of the traditional enteric media, the most widely used is MacConkey (MAC) agar (13, 35, 39, 140). Cefsulodin-irgasan-novobiocin (CIN) agar (128) and *Salmonella-Shigella* deoxycholate calcium chloride (SSDC) agar (156) were developed specifically for the isolation of *Y. enterocolitica.* These are the two most commonly used media for food samples (39, 68, 112a). CIN agar is the most acceptable agar for clinical samples because of the high confirmation rate of presumptive isolates and its high selectivity for fecal specimens (4, 61). However, most strains of *Y. enterocolitica* of pathogenic bioserotype 3/O:3 are inhibited when samples are inoculated onto CIN medium (50). Three other selective agars, BABY4 (7), virulent *Yersinia enterocolitica* (VYE) (49), and KV202 (71) agars, developed for isolation of *Y. enterocolitica,* are not extensively used. Statens Serum Institute (SSI, Copenhagen, Denmark) enteric medium, a universal medium for recovery of enteric pathogens, is effective in detecting *Yersinia* spp. in fecal samples (17).

Identification

Devenish and Schiemann (32) determined that a minimum of two biochemical tests, the Kligler iron and Christensen urea tests, were required to identify *Yersinia* among bacteria with similar colony morphology on CIN agar. *Y. enterocolitica* can be identified by biochemical tests such as fermentation of sucrose, rhamnose, and melibiose (133). Commercial rapid identification tests provide suitable alternatives to conventional tube tests (93, 98, 111). The API 20E system, widely used for identification of presumptive *Yersinia* isolates, is accurate in identifying *Y. enterocolitica* (5, 111, 136). This kit system has a positive identification rate of 93% for *Y. enterocolitica* incubated at 28°C instead of 37°C (5).

Pathogenicity

Assessing the pathogenicity of *Y. enterocolitica* isolates, although the majority of isolates recovered from asymptomatic carriers, food, and environmental samples are nonpathogenic and have no clinical significance (76), is important since a correlation has been found between the serotype and biotype of this species and the ability to cause infection. Serotyping by using commercial O:3, O:5, O:8, and O:9 antisera has been used extensively; however, these antigens can sometimes be found in nonpathogenic *Y. enterocolitica* strains and even in various *Yersinia* species (3). The biotyping scheme proposed by Wauters et al. (159) has been universally adopted. Pathogenic isolates can be differentiated from nonpathogenic isolates with the pyrazinamidase test (74), which is included in this biotyping scheme.

A number of phenotypic characteristics associated with the virulence plasmid have been described. Calcium dependence, measured by growth restriction on magnesium oxalate agar (14, 54), autoagglutination at 35 to 37°C (138), and uptake of Congo red (117, 120) and crystal violet (12) are the most popular indirect markers for identifying pathogenic isolates of *Y. enterocolitica*.

Because phenotypic tests are time-consuming and are not always reliable, DNA-based methods have been developed for pure culture. Several colony hybridization (31, 66, 120, 122) and PCR (1, 41, 65, 103, 118, 163) assays have been designed to verify the pathogenicity of *Y. enterocolitica* isolates specifically and rapidly. The methods are based on specific segments of the virulence plasmid (103, 163) or the chromosomal DNA (31, 41, 84, 103, 122, 155) that have known virulence functions. Aarts et al. (1) designed a duplex-PCR assay to simultaneously detect *Y. enterocolitica* and discriminate between pathogenic and nonpathogenic strains by using primers derived from enterobacterial repetitive intergenic consensus sequences.

COLONY HYBRIDIZATION METHODS

Isolation of pathogenic *Y. enterocolitica* is laborious, taking up to 4 weeks. Thus, several investigations have been undertaken to develop rapid and reliable methods for detection of pathogenic *Yersinia* strains from clinical, food, and environmental samples. By using DNA colony hybridization assays, pathogenic *Y. enterocolitica* strains are detected using gene probes targeting the virulence plasmid (70, 100) or virulence-related DNA sequences in the chromosome (37, 55).

Probes based on nucleotide sequences from the *virF* and *yadA* genes on the virulence plasmid have been used by Kapperud et al. (79), Nesbakken et al. (109), and Weagant et al. (160). The regulatory *virF* gene plays a major role in the coordinated thermal induction of virulence determinants (21), while the *yadA* gene encodes a multifaceted outer membrane protein, YadA, which is an essential virulence factor of *Y. enterocolitica* (38). Goverde et al. (55) designed a colony hybridization method using probes targeting the chromosomal *ail* and *inv* genes, and Durisin et al. (37) designed a method involving the *yst* gene. *ail* codes for the membrane-associated protein Ail, which promotes invasion into eukaryotic cells and confers serum resistance (16, 102). The *inv* gene of *Y. enterocolitica* encodes a product that allows bacteria to invade epithelial cells (101). DNA homologous to the *inv* locus is found in all *Y. enterocolitica* strains, but nonpathogenic isolates do not contain functional *inv* sequences (115). The *yst* gene, found in *Y. enterocolitica* but not in *Y. pseudotuberculosis* or *Y. pestis,* encodes a heat-stable enterotoxin, Yst, thought to be involved in pathogenesis (25). Among other *Yersinia* species, *Y. kristensenii* has DNA homologous to *yst* (31).

Colony hybridization does not require isolation of pure cultures, and it enables the rapid detection and enumeration of all pathogenic bioserotypes. A high background flora does, however, reduce the efficiency of hybridization because target cells grow insufficiently strongly in the presence of a competing microflora (37). Despite this, Nesbakken et al. (109) found that the prevalence of pathogenic *Y. enterocolitica* in Norwegian pork products was substantially higher when tested by the colony hybridization method than by culturing.

PCR-BASED DETECTION METHODS

PCR is a promising method for detection of pathogens in clinical, food, and environmental samples. It is faster than colony hybridization method because growing isolated colonies before analysis is unnecessary. Different PCR assays have been designed for detection of pathogenic *Y. enterocolitica* in natural samples (Table 2).

Target Genes

Several PCR assays have been developed to detect pYV-positive *Y. enterocolitica* in clinical, food, and environmental samples. Many of these methods use primers targeting the *virF* or *yadA* gene located on pYV (Table 2). Viitanen et al. (150) applied primers specific for the virulence plasmid coding the *yopN* (*lcrE*) gene of *Y. enterocolitica* O:3. The *yopN* is involved in the control of Yop release in pathogenic *Y. enterocolitica* (26). Arnold et al. (6) formulated a PCR assay based on the *yopT* gene, which encodes for YopT, an effector protein that induces a cytotoxic effect in macrophages (69).

Because of possible plasmid loss on subculture and storage (15), PCR methods targeting chromosomal virulence genes have also been created for natural samples. The *ail* gene, located in the chromosome of pathogenic *Y. enterocolitica* strains, is the most frequently used target (Table 2). In addition, some PCR assays have been designed to detect the *inv* and *yst* genes (Table 2). Weynants et al. (161) developed a PCR method to detect *Y. enterocolitica* O:3 in fecal samples; primers were designed to amplify a fragment of the *rfbC* gene. The *Yersinia*-specific region of the 16S rRNA gene has been used to detect *Yersinia* spp., especially in blood samples (Table 2).

Numerous PCR methods have been created to detect more than one *Y. enterocolitica* gene at the same time. The most common gene combination in these multiplex PCR assays has been *virF* and *ail* (Table 2). In these methods, samples contaminated with both pYV-positive and -negative isolates can be detected simultaneously. Lantz et al. (89) invented a multiplex PCR method to concurrently detect the plasmid-borne *yadA* gene and a *Yersinia*-specific region of the 16S rRNA gene. Two multiplex PCR methods using a mixture of primers against *inv, ail,* and *virF* have been designed to detect *Y. en-*

TABLE 2. PCR methods developed for detection of *Y. enterocolitica* in clinical, food, and environmental samples

Sample	Gene region	Sample preparation	Detection system	Reference
Blood, synovial fluid	*IcrE*	Proteinase K treatment	Single PCR, agarose gel	150
Blood	*virF, ail*	Preenrichment + proteinase K	Single PCR, agarose gel	40
Feces	*yst*	DNA purification	Single PCR, agarose gel	67
Food, water	*yadA*	Preenrichment + IMS[a] + proteinase K	Nested PCR, agarose gel/colorimetric detection	79
Food	*virF, ail*	Cold enrichment for 3 wk + EDTA/DNA purification/silica purification	Multiplex PCR, agarose gel	75
Feces, tonsils	*inv*	Preenrichment + IMS + proteinase K/DNA purification	Single PCR, agarose gel/colorimetric detection	119
Feces	*virF, ail, yst*	DNA purification	Multiplex PCR, agarose gel	59
Water	*ail*	Preenrichment + DNA purification	Seminested PCR, polyacrylamide gel	126
Tonsils	*virF, ail*	Preenrichment + NaOH treatment	Nested PCR, agarose gel	144
Feces	*virF, ail, inv, rfbC*	DNA purification	Multiplex PCR, agarose gel	161
Food	*yst*	Preenrichment + Triton X-100	Single PCR, agarose gel	154
Food	*virF, ail*	Preenrichment + proteinase K	Multiplex PCR, agarose gel	10
Food	*virF, ail*	DNA purification	Multiplex PCR, agarose gel	112
Tissue, feces	16S rRNA	DNA purification	Seminested PCR, colorimetric detection	146
Food	16S rRNA, *yadA*	Preenrichment + buoyant-density gradient centrifugation	Multiplex PCR, agarose gel	89
Water, sewage	*yadA*	Preenrichment + proteinase K	Nested PCR, agarose gel	153
Feces, food	*ail*	Preenrichment + DNA purification	Single PCR, fluorogenic detection (TaqMan)	73
Food	*ail*	Preenrichment + buoyant-density gradient centrifugation + NaOH treatment	Single PCR, agarose gel	86
Food	*yst*	Preenrichment + DNA purification	Single PCR, fluorogenic detection (TaqMan)	151
Food	*yst*	Preenrichment + DNA purification	Seminested PCR, agarose gel	114a
Blood	16S rRNA	DNA purification	Single PCR, fluorogenic detection (TaqMan)	134
Feces	*yopT*	Preenrichment + DNA purification	Single PCR, agarose gel	6
Food	*yadA, ail*	Preenrichment + silica purification	Multiplex PCR, agarose gel	20
Food	*ail*	Preenrichment + DNA purification	Single PCR, fluorogenic detection (TaqMan)	20
Blood	16S rRNA	DNA purification	Multiplex PCR, fluorogenic detection (TaqMan)	135

[a] IMS, immunomagnetic separation.

terocolitica and *Y. pseudotuberculosis* in food and water (75, 103). Harnett et al. (59) developed a multiplex PCR to detect the *yst, ail,* and *virF* genes of *Y. enterocolitica* simultaneously in fecal samples. Weynants et al. (161) combined the *rfbC, inv, ail,* and *virF* genes in a multiplex PCR assay to differentiate *Y. pseudotuberculosis,* pathogenic *Y. enterocolitica,* and *Y. enterocolitica* O:3 in feces.

Sample Preparation

Although the PCR technique can be extremely effective with pure microbial cultures, its sensitivity is reduced when it is applied directly to natural samples. One important reason for this is the complex composition of such samples as feces, blood, cheese, chicken, and soil, which can inhibit PCR (88, 124). Proteinases, which destroy the DNA polymerase structure, have been suggested to be a significant group of PCR inhibitors in many biological samples (124). The PCR-inhibitory effect of feces is caused by several substances, one known group being bile salts (90, 148, 162). PCR inhibition observed in blood samples is caused mainly by heme and can be relieved by addition of bovine serum albumin (88). PCR inhibition observed in pork is caused mainly by heat-stable molecules that have passed through a 0.2-μm filter (89). Thus, different sample preparation steps have been recommended for different materials.

Several methods, including enrichment, dilution, filtration, centrifugation, and adsorption, have been used for concentration and separation of *Y. enterocolitica* strains in natural samples. An enrichment step prior to PCR, which increases sensitivity and ensures the detection of viable cells, has been applied in most procedures (Table 2). To inhibit the growth of the competing microflora, selective enrichment broth may be used (10, 20, 73, 89). Since food surfaces are the primary site of bacterial contamination, a nondestructive swabbing procedure for enrichment of *Y. enterocolitica* prior to PCR reduces the amount of nonspecific food-derived DNA and possible inhibitory factors in the food matrix (10).

A dilution step is recommended for heavily contaminated samples such as feces, food, and soil in order to reduce the number of inhibitory compounds and the high concentrations of nontarget DNA (78, 88). Waage et al. (153) have demonstrated that dilution after overnight enrichment is sometimes necessary prior to bacterial lysis to obtain a positive PCR result, presumably due to inhibition by the high DNA content in the enrichment broth.

A centrifugation step is generally used to concentrate *Y. enterocolitica* strains prior to PCR. The major drawback in centrifugation is the coconcentration of inhibitory particles together with the target organism (88). Buoyant density centrifugation is used to concentrate *Y. enterocolitica* strains and to remove PCR inhibitors (86, 89, 94). An immunomagnetic separation procedure has been used in some studies to concentrate and separate *Y. enterocolitica* O:3 from PCR inhibitors

(79, 119). Filtration has been used to concentrate *Y. enterocolitica* strains in water samples (78, 126, 153).

DNA Extraction

DNA can be extracted from the cell either by lysing the cell wall to release the DNA or by using more laborious DNA purification procedures. Heat is routinely used, prior to PCR, to break down the cell wall of microbes and inactivate heat-labile PCR inhibitors (88). However, when natural samples are studied, heat treatment alone is insufficient for *Y. enterocolitica* (75). Proteinase K treatment is most commonly used before heat treatment in the PCR methods designed for direct detection of *Y. enterocolitica* in natural samples (Table 2). Proteinase K degrades cell wall proteins and PCR-inhibitory proteins and polypeptides in the sample and prevents heat-stable DNase contamination (96). *Y. enterocolitica* possesses nuclease activity to break down the PCR product; this can be prevented by proteinase K treatment prior to PCR (9, 104). Dickinson et al. (34) have shown that by increasing the amount of proteinase K from 0.2 to 1 mg per ml and by using isopropanol precipitation of DNA, *Y. enterocolitica* can efficiently be detected directly in raw chicken and cheese samples.

DNA purification has been carried out by traditional phenol-chloroform extraction and ethanol precipitation in some PCR assays developed to detect *Y. enterocolitica* directly in natural samples (59, 112, 114a, 161). This method is, however, laborious, time-consuming, and unsuitable for large numbers of samples. Numerous commercial DNA purification kits are available to make DNA isolation faster and easier. Some of these kits have also been used in PCR assays designed for *Y. enterocolitica* (6, 11, 20, 73, 126, 134, 151).

Detection of PCR Products

The method most frequently used to detect PCR products of *Y. enterocolitica* is electrophoresis in an agarose gel (Table 2). This gives both the size and number of products and a rough estimation of the concentration. With this method, however, it is not possible to ensure that the PCR product contains the correct sequence between primers. In addition, ethidium bromide, which is a mutagen, is used to stain the agarose gel and may not be appropriate for routine use in food-monitoring laboratories. To overcome these problems, Rasmussen et al. (119) detected the amplified products of *Y. enterocolitica* by capture of the products using hybridization to an immobilized oligonucleotide. The immobilized PCR products in microtiter wells were detected with fluorescence. Recently, the 5′-nuclease PCR (TaqMan) assay, which no longer requires gel-based detection, has been used to detect *Y. enterocolitica* directly in food samples (20, 73, 151) and in blood (134). With this fluorogenic method, the probe is designed to hybridize with an internal region of the target sequence. When the probe hybridizes with its target, the reporter dye is cleaved and becomes capable of emitting a fluorescent signal that can be detected in real time. Aarts et al. (1) have developed an online rapid-cycling real-time PCR assay, using the SYBR Green I format to detect pathogenic *Y. enterocolitica* in pure culture. The double-stranded DNA of the PCR product binds the SYBR Green I dye, and no internal probes are needed.

False-Positive Results

When traditional PCR detection with several manual steps is used, false-positive results due to cross-contamination may be a problem if specific precautions have not been taken into account (60). Carryover contamination in PCR assays can also result in false-positive results. However, these false-positive results can be monitored with a sufficient number of negative controls. False-positive results can also occur if the primers are not adequately specific, especially when low annealing temperatures are used or when sequences of target genes are also found in nonpathogenic strains. Grant et al. (56) have shown that strains of *Y. enterocolitica* of nonpathogenic biotype 1A can sometimes carry sequences homologous to the chromosomal virulence-associated genes *ail, myf,* and *yst.* False-positive results due to dead cells can be avoided by using an enrichment step prior to PCR. This ensures the detection of viable cells and increases sensitivity. Rasmussen et al. (119) have demonstrated that an enrichment step is needed before PCR to increase sensitivity when naturally contaminated samples are studied. A preenrichment step was also used in most procedures where pathogenic *Y. enterocolitica* was detected in natural samples (Table 2).

False-Negative Results

False-negative PCR results may occur more often than false-positive results when natural samples are studied (45, 46, 72). The main reason for false-negative results is the presence of inhibitor factors in food samples (88, 124). Enrichment media can also interfere with PCR detection; even a small amount of $MgCl_2$, which is found in both ITC and MRB broths, can be PCR inhibitory (124). To overcome this problem, Knutsson et al. (81) developed a PCR-compatible enrichment medium for *Y. enterocolitica* that makes sample treatment before PCR unnecessary. However, no easy method for overcoming PCR inhibition caused by natural samples has yet been discovered. False-negative results caused by inhibitory substances in the sample can be monitored by using an internal positive control (143). False-negative results can sometimes be caused by sequence heterogeneity of target genes between different *Y. enterocolitica* strains (20, 110).

OCCURRENCE IN NATURAL SAMPLES

Clinical Samples

Animals have long been suspected of being reservoirs for *Y. enterocolitica* and, hence, sources of human infection. Numerous studies have been carried out to isolate *Y. enterocolitica* strains from a variety of animals (64). However, most of the strains isolated from animal sources differ both biochemically and serogically from strains isolated from humans with yersiniosis. Human-pathogenic strains of *Y. enterocolitica* have frequently been isolated only from tonsils and fecal samples from slaughtered pigs (133).

Cold enrichment has commonly been used when studying samples from slaughtered pigs (Table 3). However, selective enrichment in ITC or MRB is clearly more productive than cold enrichment, especially when tonsils and mesenteric nodes were studied (29, 30, 43). These selective liquid media have

TABLE 3. Detection of pathogenic *Y. enterocolitica* in slaughtered pigs by culture methods

Sample	No. of samples	No. of samples positive for:				Culture method[a]	Reference
		O:3	O:5,27	O:8	O:9		
Throat	1,200	86	1			CE	52
Feces	1,200	88	1			CE	52
Mesenteric lymph nodes	200					CE	52
Tonsils	86	33	3			SE + CE	29
Feces	100	16			1	SE + CE	29
Tonsils	202	57	18			SE + CE	58
Tonsils	106	43			2	D + SE + CE	30
Mesenteric lymph nodes	108	17				D + SE + CE	30
Throat	3,375	4	96[b]			CE	53
Feces	1,420	235		1	9	CE	92
Tonsils	291	67	1		2	CE	142
Feces	291	17				CE	142
Tonsils	50	30				D + ON + SE + CE	46
Feces	50	5				D + ON + SE + CE	46

[a] CE, cold enrichment; SE, selective enrichment; ON, overnight enrichment; D, direct plating.
[b] *ail*-positive serotype O:5.

been designed specifically for isolation of serotype O:3. MRB is inhibitory to some other pathogenic serotypes, particularly O:8 (130). In addition, De Zutter et al. (33) have shown that ITC is not optimal for recovery of serotype O:9. These problems can be overcome with nonselective or poorly selective cold enrichment. However, nonpathogenic isolates and other psychrotrophic bacteria also multiply during cold enrichment. Funk et al. (53) isolated *Y. enterocolitica* from 808 throat samples, only 107 of which harbored *ail*-positive *Y. enterocolitica.* Overnight enrichment at room temperature in nonselective broth provides an alternative to cold enrichment in finding asymptomatic carriers. Fredriksson-Ahomaa et al. (43) recovered pathogenic *Y. enterocolitica* strains from all positive fecal samples after overnight enrichment in TSB. An enrichment step is generally needed for asymptomatic carriers, while direct plating is usually sufficient for patients with diarrhea because the number of *Y. enterocolitica* organisms excreted by the latter is relatively large (4). In Belgium, most laboratories have stopped using cold enrichment since it also increases the isolation of nonpathogenic *Y. enterocolitica* strains (149).

Studies have been conducted to compare culture and PCR methods for their ability to detect pathogenic *Y. enterocolitica* in tonsil and fecal samples (see Table 5). The detection rate was shown to be significantly higher with PCR, especially when fluorogenic 5′-nuclease PCR (TaqMan) assay was used (20). Boyapalle et al. (20) have reported that the TaqMan assay was 1,000 to 10,000 times more sensitive than the culture method or traditional PCR assay when fecal and tonsillar samples were studied. Sensitive methods are particularly necessary to detect pathogenic *Y. enterocolitica* in asymptomatic carriers, e.g., to study possible animal reservoirs for this pathogen. Rapid and sensitive methods are also needed to detect small numbers of *Y. enterocolitica* organisms and other bacteria in blood units used for transfusion or in asymptomatic blood donors (40, 135).

Food Samples

Food has often been suggested to be the main source of *Y. enterocolitica* infection, although pathogenic isolates have seldom been recovered from food samples (28, 48, 113). Raw pork products have been widely investigated because of the association between *Y. enterocolitica* 4/O:3 and pigs. However, the isolation rate of pathogenic bioserotypes of *Y. enterocolitica* has been low in raw pork except for pig offal, with the most common type isolated being bioserotype 4/O:3 (Table 4). In these studies, selective enrichment in ITC and MRB has mostly been used. *Y. enterocolitica* strains belonging to bioserotypes associated with human disease have been recovered only a few times from beef, poultry, and milk samples (2, 51, 95). In these cases, cross-contamination has probably occurred during processing, packing, or handling since pathogenic *Y. enterocolitica* strains have thus far never been recovered from live cattle or poultry.

The occurrence of pathogenic *Y. enterocolitica* in some foods has been estimated by both culture and PCR methods (Table 5). In all of these studies, the prevalence was clearly higher by PCR than by culturing, showing the higher sensitivity of the former for naturally contaminated samples. For artificially contaminated pork, Boyapalle et al. (20) have shown that the TaqMan assay targeting the *ail* gene was 100 to 1,000 times more sensitive than the traditional PCR assay with gel-based detection and 10,000 times more sensitive than the culture method. Visnubhatla et al. (152) used the same TaqMan assay, but instead of *ail,* they targeted the *yst* gene. This was the first time when a high occurrence of *yst*-positive *Y. enterocolitica* was detected in retail ground beef. In the same study, the isolation rates of *Y. enterocolitica* in ground beef and pork were also elevated. The contamination level of these products was obviously very high, because the culture method used was able to identify *Y. enterocolitica* only when 10^6 CFU or more organisms per g were present (152). The occurrence of pathogenic *Y. enterocolitica* was also shown to be clearly higher by the PCR assay than by culturing (Table 5).

Environmental Samples

Most of the *Y. enterocolitica* isolates recovered from environmental samples, including the slaughterhouse, fodder, soil, and water, have been nonpathogenic (8, 22, 24, 97, 125, 140). However, strains of bioserotype 4/O:3 have occasionally been

TABLE 4. Detection of pathogenic *Y. enterocolitica* in pork products by culture methods

Sample	No. of samples	No. of samples positive for:				Country of origin of sample	Reference (number)
		O:3	O:5,27	O:8	O:9		
Tongue	302	165			3	Belgium	157
	37	11				Canada	129
	31	2		6		United States	36
	47	26				Norway	106
	50	20				Japan	137
	125	8				Spain	57
	29	28				Belgium	158
	40	6			2	The Netherlands	29
	55	14				Germany	80
	86	2				Italy	30
	99	79				Finland	48
	20	15				Germany	43
Offal[a]	34	17				Finland	47
	16	5				Finland	48
	100	46				Germany	43
Pork[b]	91	1		1		Canada	129
	127	1				Norway	108
	70	22			3	Japan	137
	267	6				Denmark	23
	50	12				Belgium	158
	400	3			1	The Netherlands	29
	45	8				Norway	109
	67	1	8[c]	3		China	147
	48	1			1	Germany	80
	40	2	4		1	Ireland	95
	1,278	64	14			Japan	51
	255	4				Finland	45
	300	6				Norway	72
	120	14				Germany	43

[a] Offal, excluding tongue.
[b] Other pork products, excluding offal.
[c] Isolates belonging to serotype O:5 and showing autoagglutination activity and calcium-dependent growth.

TABLE 5. Detection of pathogenic *Y. enterocolitica* in natural samples by PCR and culture methods

Sample	No. of samples			Reference
	Total	Culture positive[a]	PCR positive	
Clinical				
Pig tonsils	185	48	58	46
Pig tonsils	252	0	90	20
Pig feces	255	0	80	20
Mesenteric lymph nodes	257	0	103	20
Food				
Pig tongues	51	40	47	45
Minced pork	255	4	63	45
Pig offal	34	17	21	47
Chicken	43	0	0	48
Fish	200	0	0	48
Lettuce	101	0	3	48
Pork[b]	300	6	50	72
Pig tongues	15	7	10	152
Ground pork	100	32	47	152
Ground beef	100	23	31	152
Tofu	50	0	6	152
Ground pork	350	0	133	20
Chitterling	350	8	278	20
Environmental				
Water	105	1	11	126
Slaughterhouse	89	5	12	47

[a] The pathogenicity of the isolates has been confirmed.
[b] Except pig tongues and offal.

isolated from the slaughterhouse (42, 107) and sewage water (23). Sandery et al. (126) and Fredriksson-Ahomaa et al. (47) have shown that compared with traditional PCR assays, culture methods underestimate the occurrence of pathogenic *Y. enterocolitica* in environmental samples (Table 5).

CONCLUSIONS

Inefficient isolation methods have been the predominant reason for the low prevalence rates of pathogenic *Y. enterocolitica* in earlier studies; the detection limit for pathogenic *Y. enterocolitica* is 10^3 to 10^6 CFU or more organisms per g in feces and pork samples. Thus, *Yersinia*-selective agar plates are insufficiently sensitive. In addition, nonpathogenic *Y. enterocolitica* colonies have the same appearance as pathogenic ones, which makes it difficult to select appropriate colonies for confirmation. While selective enrichment media are not selective enough, they do contain agents which inhibit the growth of some pathogenic strains. Thus, one reason why the most frequently recovered *Y. enterocolitica* strain is bioserotype 4/O:3 might be that the isolation methods favor this bioserotype.

PCR assays have provided a better estimation of the occurrence of pathogenic *Y. enterocolitica* in clinical, food, and environmental samples than have culture methods. Real-time PCR assays are rapid and sensitive methods for identification and enumeration of pathogenic *Y. enterocolitica* in natural samples. Future improvements in real-time PCR assays that focus on incorporating positive internal controls and increasing the automation of the entire process will enhance the usefulness of this method in laboratory diagnostic and epidemiological studies, and the food industry. When sampling becomes automated, the sample-handling time will be reduced and cross-contamination will be minimized. However, effective isolation methods are also needed, because without isolation of *Y. enterocolitica* strains, no strain characterization can be performed, and with no strain characterization, important epidemiological information will be missing.

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