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Detection, Semiquantitative Enumeration, and Antimicrobial Susceptibility of *Yersinia enterocolitica* in Pork and Chicken Meats in Italy

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ABSTRACT

Yersinia enterocolitica is recognized as an etiological agent of gastroenteritis, lymphadenitis, and chronic sequelae. During 2006 and 2007, 205 samples (125 pork and 80 chicken meats) were collected in Italy and tested for detection and most-probable-number (MPN) enumeration of *Y. enterocolitica* organisms. The microorganism was isolated from 45 samples (21.9%): 19 (15.2%) pork samples and 26 (32.5%) chicken samples. *Y. enterocolitica* MPN contamination levels were low, ranging from 0.30 to 1.50/g. Most (94.4%) *Y. enterocolitica* strains were biotype 1A (serotypes O:3; O:5; O:6,30; O:6,30-6,31; O:7,8-8-8,19; O:8; O:9; O:25,35; O:36; and O nontypeable), and 5.6% of the isolates were bioserotype 2/O:9. All isolates were tested for *yadA, ail, inv, ystA,* and *ystB* virulence sequences. The *yadA* gene was detected in two strains (3.7%) isolated from chicken samples: one *Y. enterocolitica* 2/O:9 $yadA^+$ ail^+ $ystA^+$, and one *Y. enterocolitica* 1A/O:7,8-8-8,19 $yadA^+$ inv^+ $ystB^+$. Two (3.7%) 2/O:9 strains, isolated from pork products, were ail^+ $ystA^+$. Most biotype 1A strains were $ystB^+$ (84.3%) and inv^+ (39.2%). All strains were sensitive to cefotaxime, ciprofloxacin, chloramphenicol, nalidixic acid, streptomycin, sulfonamide, tetracycline, trimethoprim, and trimethoprim-sulfamethoxazole. Resistance to gentamicin and aztreonam was observed in 1.9% of the isolates. High levels of resistance were detected toward amoxicillin–clavulanic acid (27.8%), ampicillin (75.9%), and erythromycin (100%). The authors hypothesize that *Y. enterocolitica* pathogenic biotypes are rather uncommon in foods when compared with their isolation rates from animal sources and that chicken meat could be contaminated as well as pig meat and its derived products.

Yersinia enterocolitica is a food- and waterborne human pathogen, able to cause a wide spectrum of diseases, ranging from acute diarrhea, terminal ileitis, and mesenteric lymphadenitis to long-term sequelae such as reactive arthritis and erythema nodosum *(15)*. In children, severe manifestations of peritonitis and pseudoappendicitis have been reported *(14, 65)*. Septicemia caused by *Y. enterocolitica* may occur in otherwise healthy as well as in immunocompromised people, or in hosts suffering from underlying disorders, such as iron overload *(11)*. Immunologically induced sequelae such as reactive arthritis (most common), erythema nodosum, Reiter's syndrome, glomerulonephritis, and myocarditis are observed, especially in Scandinavians *(11)*. Reactive arthritis cases occur especially in HLA-B27–positive individuals *(23)*.

In the United States, *Y. enterocolitica* human infection is rare, with an average annual incidence of 3.5 cases per 1,000,000 persons *(46)*; 90% of the cases are estimated to be foodborne *(49)*. In Europe, *Y. enterocolitica* is one of the main hazards identified on pig carcasses, with mean rates of prevalence higher than 30% *(29)*. Data on *Y. enterocolitica* contamination rates in foodstuffs are normally related to pig meat and products thereof, as the pig is the most important reservoir of the microorganism *(15),* but other meats are seldom investigated. Milk and dairy products are mainly contaminated by nonpathogenic *Y. enterocolitica* isolates. In such products pathogenic strains are mostly linked with outbreaks of yersiniosis *(3, 5)*.

The major goal of this study was the evaluation of the prevalence of *Y. enterocolitica* in pork and chicken meat samples collected at retail level in Italy, together with the quantification of its contamination level by using a three-tube most-probable-number (MPN) method. Since *Y. enterocolitica* strains carry different degrees of pathogenicity *(4),* all isolates were tested for plasmid- and chromosome-borne virulence genes. The plasmid-borne virulence gene analyzed was *yadA,* whose product is involved in serum resistance, autoagglutination, and attachment of *Y. enterocolitica* to the intestinal brush border *(55, 64)*. The chromosomal virulence genes included in the analysis were *ail, inv, ystA,* and *ystB*. The *ail* (named for adhesion invasion locus) gene encodes a surface factor that enhances epithelial cell penetration *(50),* and the *inv* gene encodes the

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major invasive factor of *Y. enterocolitica (60)*. The *ystA* and *ystB* genes encode two heat-stable enterotoxins. *Yersinia* stable toxin a (YST-a) is produced by enteropathogenic strains of *Y. enterocolitica* and may contribute to the pathogenesis of diarrhea associated with acute yersiniosis *(22)*. YST-b is an enterotoxin produced mainly by biotype 1A of *Y. enterocolitica (58)*.

In addition, according to Directive EC 2003/99 on zoonosis and zoonotic agents monitoring programs, the antibiotic resistance of *Y. enterocolitica* isolates was evaluated.

MATERIALS AND METHODS

Sample collection. From January 2006 to December 2007, 125 samples of raw pork products and 80 samples of raw chicken meats were collected at retail level from 55 stores in Emilia-Romagna, Lombardia, and Trentino Regions, Northern Italy. The pork samples were represented by 55 sausages, 40 raw meat samples, 20 minced meats, and 10 short-ripened salami. The chicken meat samples were represented by 75 raw meat samples (portions of breast, legs, and wings) and 5 minced meats. All specimens were transported at 3 ± 2°C to the laboratory and stored at 3 ± 2°C for 2 to 18 h before being analyzed.

Detection of *Y. enterocolitica*. A 25-g amount of sample was suspended 1:10 in phosphate-buffered saline with 2% sorbitol and 1.5% bile salts (PSB; Biolife Italiana, Milan, Italy). The PSB broth was incubated for 2 h at room temperature (resuscitation) before a 10-μl aliquot was plated onto cefsulodin-irgasan-novobiocin (CIN) *Yersinia*-selective agar (Oxoid, Basingstoke, UK) and incubated at 30 ± 1°C for 48 h. The PSB suspensions were stored at 5 ± 3°C for 5 weeks (cold enrichment) and streaked onto CIN agar plates weekly. Flat colonies with an entire edge and a red center (''bull's-eye'') surrounded by a translucent, transparent, or milk-white zone were considered suspect *Y. enterocolitica* colonies. As colony morphology may vary among *Y. enterocolitica* biotypes, even circular, erose-edged colonies showing a red center were selected for confirmation *(40)*. Confirmation tests were performed by seeding colonies in Kligler iron agar (bioMérieux, Marcy l'Etoile, France) and Christensen's urea agar (LAB-M, Bury, UK), which were incubated at 30 ± 1°C for 48 h.

Y. enterocolitica species identification was performed using the API 20E system (bioMérieux) incubated at 30 ± 1°C for 48 h. All *Y. enterocolitica* isolates were stored at 3 ± 2°C before biotyping, serotyping, and genotyping.

MPN enumeration of *Y. enterocolitica*. For the enumeration of *Y. enterocolitica,* a three-tube MPN method based on cold enrichment was tested. The method was first validated in our laboratory by using spiked samples and evaluating sensitivity and specificity. Briefly, 10 raw beef samples were contaminated with a *Y. enterocolitica* bioserotype 1A/O:5 strain (code 116; Institute of Health, Rome, Italy). Inoculum levels ranged from 10 to 10^2 CFU/g. Two groups of five samples each were inoculated with 10 *Y. enterocolitica* CFU/g and 10^2 CFU/g, respectively. Five non-inoculated raw beef samples were used as negative controls. All samples were suspended 1:10 in PSB. The three-tube MPN dilutions were prepared in PSB and represented 1.0, 0.1, and 0.01 g of the samples. On the day of inoculation, 0.1 μl from each dilution tube was plated onto CIN agar. The day of inoculum represented day zero. The dilution series were stored at 5 ± 3°C for 5 weeks, and a 0.1-μl aliquot of each tube was plated onto CIN agar weekly (for a total number of CIN agar plates of nine plates per sample and per week). Incubation and colony selection were performed as previously described. When present, up to five suspect colonies per plate were selected for biochemical and serological confirmation. All raw beef samples used to validate the cold-enrichment MPN method were tested for aerobic mesophilic bacterial count, following the ISO 4833 standards. Aerobic mesophilic flora values ranged from 3.2×10^6 to 7.5×10^7 CFU/g. All but one spiked beef sample gave positive results by the MPN method. The false-negative sample had been contaminated with 10 *Y. enterocolitica* organisms per g, but no colonies were detected on CIN agar plates even after 5 weeks of cold enrichment. In the remaining *Y. enterocolitica*–spiked samples, the microorganism was always detectable on day zero (90.0%). Thereafter, isolation was negligible during the first and second weeks of cold enrichment, with abundant background flora growing on CIN agar plates. *Y. enterocolitica* was reisolated after 3 (50%) or 4 (40%) weeks of cold enrichment. MPN values were lower than inoculum levels in all spiked samples. In samples contaminated with 10 CFU/g, *Y. enterocolitica* MPN values ranged from 0.4 to 1.5/g (mean value, 0.75/g), whereas in samples contaminated with 100 CFU/g, *Y. enterocolitica* MPN values ranged from 3.6 to 16.0/g (mean value, 9.2/g). In negative controls, *Y. enterocolitica* populations were <0.30/g. According to our data, sensitivity and specificity of the cold-enrichment MPN method were 90.0 and 100.0%, respectively. In our small survey, *Y. enterocolitica* MPN enumeration in the pork and chicken meat samples was performed following the proposed cold-enrichment MPN method.

Biotyping. The *Y. enterocolitica* strains were biotyped according to the modified Wauters' scheme *(15)*.

Serotyping. Serotyping was performed by slide agglutination with commercially available O antisera for the serogroups involved in most cases of human yersiniosis in Europe: O:1-2, O:3, O:5, O:8, and O:9 (Denka Seiken, Tokyo, Japan). O nontypeable strains were sent to the Institut Pasteur, Unité des *Yersinia* (Paris, France), for typing.

Detection of virulence genes. *Y. enterocolitica* isolates were tested by PCR assays for plasmid- and chromosome-borne virulence genes. To detect the plasmid virulence (pYV)–positive *Y. enterocolitica* strains, a PCR method using primers targeting the *yadA* gene was followed, using a *Y. enterocolitica* strain bioserotype 4/O:3 (code CIP-864; Centre National de Référence des *Yersinia,* Institut Pasteur, Paris, France) as positive control *(10)*. Isolates were subcultured four or five times to obtain pure colonies and to perform biochemical tests. Because of possible plasmid loss on repeated laboratory cultures and storage at 5 ± 3°C, PCR assays targeting the chromosomal virulence *ail, inv, ystA,* and *ystB* genes were also performed. For the *ail* gene detection, a simplex-PCR method was followed *(10),* using a *Y. enterocolitica* strain bioserotype O:5,27 (code 837; Institute of Public Health, Oslo, Norway) as positive control. A multiplex-PCR assay designed for the simultaneous detection of the *inv, ystA,* and *ystB* sequences was applied *(69),* using a 1A/O:5 *Y. enterocolitica* strain (code 116; Institute of Health, Rome, Italy) as positive control.

Resistance to antimicrobial agents. *Y. enterocolitica* was stored at −80°C with 20% glycerol in cryogenic vials before being tested for antimicrobial resistance by the disk diffusion method according to the NCCLS (now Clinical and Laboratory Standards Institute) *(52),* except that the incubation temperature was 30°C. Iso-Sensitest agar (Oxoid) and commercial antimicrobial suscep-

tibility disks (Oxoid) were used. The following 14 antimicrobial agents were selected: ampicillin (10 μg), amoxicillin–clavulanic acid 2:1 (30 μg), aztreonam (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), erythromycin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfonamide (300 μg), tetracycline (30 μg), trimethoprim (5 μg), and trimethoprim-sulfamethoxazole (1.25 and 23.75 μg). Results were interpreted according to international standards *(52)*.

RESULTS

Detection and MPN enumeration of *Y. enterocolitica* in pork meats. A total of 19 (15.20%) of 125 raw pork meat and pork products samples were contaminated by *Y. enterocolitica*. The highest contamination rates (20.0%) were observed for raw meat and minced meat, respectively, followed by fresh sausages (10.9%) and short-ripened salami (10.0%).

Five (26.3%) of 19 samples gave a positive result by the detection method and 17 (89.5%) by the MPN method. The two methods were not in agreement, as only 3 (15.8%) samples were positive by both techniques, while 2 (10.5%) samples were positive only by the detection method and 14 (73.7%) samples were positive only according to MPN analysis. In the pork samples, the MPN values for *Y. enterocolitica* ranged from 0.30 to 1.5/g.

A total of 22 *Y. enterocolitica* isolates were detected, as three specimens were contaminated by different strains. According to our data, 20 strains (90.9%) were biotype 1A and 2 strains (9.1%) were biotype 2 (serotype O:9). Of the biotype 1A isolates, 11 (55.0%) were serotype O:5, 1 (5.0%) was serotype O:7,8-8-8,14, 3 (15.0%) were serotype O:8, 1 (5.0%) was serotype O:9, and 4 (20.0%) were O nontypeable (ONT). The estimated MPN values of the two pathogenic 2/O:9 strains were 0.30/g (a minced-meat sample) and 0.92/g (a fresh sausage).

The *yadA* plasmid-borne gene was never detected. The *Y. enterocolitica* 2/O:9 isolates were positive for both the *ail* and the *ystA* genes. Among *Y. enterocolitica* biotype 1A, six (30.0%) isolates carried both the *inv* and the *ystB* genes, one (5.0%) carried both the *ystA* and the *ystB* sequences, but the majority of the strains (90.0%) carried the *ystB* gene only. Two isolates lacked the virulence genes tested (Table 1).

Detection and MPN enumeration of *Y. enterocolitica* in raw chicken meats. A total of 26 (32.5%) of 80 chicken meat samples were contaminated by *Y. enterocolitica*. Seven (26.9%) of 26 samples gave a positive result by the detection method and 23 (88.4%) by the MPN method. Only 4 (15.4%) samples were positive by both techniques, 3 (11.5%) samples were positive by the detection procedure only, and 20 (76.9%) by MPN analysis only. In the chicken meat samples, the MPN values for *Y. enterocolitica* ranged from 0.30 to 0.92/g.

A total of 32 *Y. enterocolitica* isolates were detected, as some samples were contaminated by more than one strain. Of 32 strains, 31 (96.9%) were biotype 1A, and 1 (3.1%) was biotype 2 (O:9). Among the 31 biotype 1A isolates the following serotypes were detected: O:3 (3.2%), O:5 (19.4%), O:8 (22.6%), O:6,30 (3.2%), O:6,30-6,31 (3.2%), O:25,35 (3.2%), O:36 (3.2%), and O:7,8-8-8,19 (3.2%). Thirteen (41.9%) biotype 1A strains were ONT. The MPN contamination level of the pathogenic 2/O:9 isolate was 0.30/g.

The distribution of virulence genes in the *Y. enterocolitica* strains is shown in Table 1. The *yadA* plasmid-borne gene was detected in two isolates (6.2%): (i) *Y. enterocolitica* $yadA^+$ ail^+ $ystA^+$ bioserotype 2/O:9; and (ii) *Y. enterocolitica* $yadA^+$ inv^+ $ystB^+$ bioserotype 1A/O:7,8-8-8,19. Three isolates (9.4%) carried the *inv, ystA,* and *ystB* genes, 7 isolates (21.9%) were inv^+ $ystB^+$, three strains (9.4%) were inv^+, 14 isolates (43.8%) were $ystB^+$, and one strain (3.1%) was $ystA^+$. Two isolates (6.2%) lacked the virulence genes tested.

Resistance to antimicrobial agents. No *Y. enterocolitica* strain was resistant to cefotaxime, ciprofloxacin, chloramphenicol, nalidixic acid, streptomycin, sulfonamide, tetracycline, trimethoprim, and sulfamethoxazole-trimethoprim. Resistances to the following antibiotics were reported: (i) aztreonam, one (1.9%) 2/O:9 ail^+ $ystA^+$ strain of pork origin; (ii) gentamicin, one (1.9%) 1A/ONT ail^- $yadA^-$ inv^- $ystA^-$ $ystB^-$ strain of poultry origin; (iii) amoxicillin–clavulanic acid, 15 (27.8%) strains, comprising the 2/O:9 $yadA^+$ ail^+ $ystA^+$ isolate of poultry origin, 5 strains of biotype 1A inv^+ $ystB^+$, and 9 strains of biotype 1A $ystB^+$; (iv) ampicillin, 41 (75.9%) strains, comprising one strain 2/O:9 $yadA^+$ ail^+ $ystA^+$ of poultry origin, and one 2/O:9 strain ail^+ $ystA^+$ of pork origin. All strains (100%) were resistant to erythromycin (Table 2).

The distribution of the antimicrobial resistance in association with the presence of virulence genes demonstrates that the highest resistance rate to ampicillin and amoxicillin–clavulanic acid was detected in genotype $ystB^+$ (80.0 and 56.3%, respectively) and in genotype inv^+ $ystB^+$ isolates (83.3 and 41.7%, respectively). All $yadA^-$, ail^-, inv^-, $ystA^-$, $ystB^-$ strains (100%) were resistant both to ampicillin and to erythromycin.

The antimicrobial resistance pattern of the 2/O:9 human pathogenic *Y. enterocolitica* isolates comprises ampicillin (66.7%), amoxicillin–clavulanic acid (33.3%), aztreonam (33.3%), and erythromycin (100%). In biotype 1A isolates, 76.5% of the strains were resistant to ampicillin, 27.5% to amoxicillin–clavulanic acid, 2.0% to gentamicin, and 100% to erythromycin.

Seven (21.9%) *Y. enterocolitica* strains isolated from chicken meat were susceptible to all antimicrobials but erythromycin, whereas the same susceptibility pattern was detected only in three (13.6%) strains isolated from pork. In *Y. enterocolitica* strains of pork origin, higher resistance rates to ampicillin (81.8 versus 71.9%) and to amoxicillin–clavulanic acid (40.9 versus 18.8%) were also observed (Table 2). No *Y. enterocolitica* strain was resistant to more than three antibiotics.

DISCUSSION

Raw pork products have often been suggested as the main source of *Y. enterocolitica* for humans, and several

TABLE 1. *Distribution of virulence genes among* Y. enterocolitica *strains isolated from pork and chicken meats*[a]

Pork meat						Chicken meat					
Bioserotype (no.) (n = 22)	Virulence genes					Bioserotype (no.) (n = 32)	Virulence genes				
	inv	*ail*	*ystA*	*ystB*	*yadA*		*inv*	*ail*	*ystA*	*ystB*	*yadA*
2/O:9 (2)	0	2	2	0	0	2/O:9 (1)	0	1	1	0	1
2 strains	−	+	+	−	−	1 strain	−	+	+	−	+
1A/O:5 (11)	3	0	1	11	0	1A/O:5 (6)	4	0	1	5	0
7 strains	−	−	−	+	−	2 strains	−	−	−	+	−
3 strains	+	−	−	+	−	2 strains	+	−	−	+	−
1 strain	−	−	+	+	−	1 strain	+	−	+	+	−
						1 strain	+	−	−	−	−
1A/O:8 (3)	1	0	0	3	0	1A/O:8 (6)	2	0	0	6	0
2 strains	−	−	−	+	−	4 strains	−	−	−	+	−
1 strain	+	−	−	+	−	2 strains	+	−	−	+	−
1A/O:9 (1)	0	0	0	0	0	1A/O:3 (1)	1	0	0	1	0
1 strain	−	−	−	−	−	1 strain	+	−	−	+	−
1A/O:7,8-8-8,19 (1)	0	0	0	1	0	1A/O:36 (1)	1	0	0	0	0
1 strain	−	−	−	+	−	1 strain	+	−	−	−	−
						1A/O:7,8-8-8,19 (1)	1	0	0	1	0
						1 strain	+	−	−	+	+
						1A/O:6,30 (1)	1	0	0	1	0
						1 strain	+	−	−	+	−
						1A/O:6,30-6,31 (1)	0	0	0	1	0
						1 strain	−	−	−	+	−
						1A/O:25,35 (1)	0	0	0	1	0
						1 strain	−	−	−	+	−
1A/ONT (4)	2	0	0	3	0	1A/ONT (13)	4	0	3	9	0
1 strain	−	−	−	+	−	6 strains	−	−	−	+	−
2 strains	+	−	−	+	−	2 strains	+	−	+	+	−
1 strain	−	−	−	−	−	1 strain	+	−	−	−	−
						1 strain	−	−	+	−	−
						2 strains	−	−	−	−	−
						1 strain	+	−	−	+	−
Total	6	2	3	18	0		14	1	5	25	2

[a] −, gene absent; +, gene present.

studies were made to assess the prevalence of *Y. enterocolitica* in pig tonsils *(13, 34, 44),* lymph nodes *(53),* intestinal content *(8, 13, 48, 51),* pork carcasses *(13, 33, 57),* and pork products *(30, 41, 42).* Even though the role of poultry in the epidemiology of human yersiniosis has so far not been investigated, recent studies have detected *Y. enterocolitica* on chicken carcasses *(28, 45)* and in products thereof *(21, 59).*

Most *Y. enterocolitica* strains associated with human yersiniosis in Europe belong to biotype 4 (serotype O:3) and biotype 2 (serotype O:9) *(24).* In Italy, *Y. enterocolitica* bioserotype 2/O:9 strains were isolated from human clinical samples, but not from foods *(27).* In our study, one *Y. enterocolitica* strain belonging to bioserotype 2/O:9, carrying the *yadA, ail,* and *ystA* genes, was isolated from a chicken meat sample, and two 2/O:9 ail^+ $ystA^+$ strains were detected in pork products represented by a minced meat sample and a fresh (not dry-cured) sausage. The prevalence of *Y. enterocolitica* enteropathogenic bioserotypes in pork and chicken meat samples was 1.6 and 1.3%, respectively. Meat contamination values by 2/O:9 *Y. enterocolitica* were very low, ranging from 0.30/g (pork minced meat and chicken meat) to 0.92/g (pork fresh sausage). Due to the psychrotrophic nature of *Y. enterocolitica,* strains present in meat have the potential to multiply during storage at refrigeration temperatures, even if they are poor competitors in comparison with the high number of psychrotrophic bacteria normally present in meat. Their ability to multiply increases at temperatures higher than 5°C and on meat with a high pH *(24),* such as chicken meat.

Enumeration of *Y. enterocolitica* in foods has seldom been performed for lack of reliable, time-saving methods. By adapting a multiplex PCR targeting the *ail* and *virF* genes to the enrichment cultures of an MPN method, amounts of *Y. enterocolitica* ranging between 0.30 and 5.42 MPN/cm^2 were detected from 32% of packs of raw pork meat in New Zealand. A presumptive result was obtained

TABLE 2. *Antimicrobial resistance in* Y. enterocolitica *isolates derived from pork and chicken meats*

Source and antimicrobial agent	No. of strains with bioserotype:											Total no. (%)
	2/O:9	1A/O:3	1A/O:5	1A/O:8	1A/O:9	1A/O:36	1A/O:6,30-6,31	1A/ O:7,8-8-8,19	1A/O:25,36	1A/O:6,30	1A/ONT	
Pork meat	2	0	11	3	1	0	0	1	0	0	4	
Erythromycin	2		11	3	1			1			4	22 (100)
Ampicillin	1		9	2	1			1			4	18 (81.8)
Amoxicillin–clavulanic acid	1		5	1	0			0			2	9 (40.9)
Aztreonam	1		0	0	0			0			0	1 (4.5)
Gentamicin	0		0	0	0			0			0	0
Chicken meat	1	1	6	6	0	1	1	1	1	1	13	
Erythromycin	1	1	6	6		1	1	1	1	1	13	32 (100)
Ampicillin	1	1	2	3		0	1	1	0	1	13	23 (71.9)
Amoxicillin–clavulanic acid	0	0	2	1		0	0	0	0	0	3	6 (18.8)
Aztreonam	0	0	0	0		0	0	0	0	0	0	0
Gentamicin	0	0	0	0		0	0	0	0	0	1	1 (3.1)

within 24 h, followed by confirmation of isolates in 18% of the samples within 4 days *(41)*.

In the present study, enumeration was performed by matching a conventional three-tube MPN method with a cold-enrichment method (mean enrichment period prior to colony growth, 4.1 weeks). Even though this MPN method is time-consuming, it demonstrated a higher sensitivity (91.3%) than the cold-enrichment detection method (26.1%). Interestingly, the pathogenic *Y. enterocolitica* 2/O:9 strains were detected both by the detection method and by the MPN procedure.

As *Y. enterocolitica* is generally present in low numbers in foods, together with a large number of organisms in the background flora, direct plating and direct count on selective media are seldom successful *(31, 54)*. No relationship was observed between the detection of *Y. enterocolitica* by traditional cold enrichment and its contamination level, given that in the 12 samples found to be positive according to the detection method the estimated MPN values ranged from 0.30 to 0.92/g. Nevertheless, although the MPN procedure was characterized by a 90.0% sensitivity in detecting artificially contaminated samples, it undoubtedly underestimated *Y. enterocolitica* contamination levels. MPN values were 1 to 2 log lower than the known inocula, and such disparities between MPN results and contamination levels could occur even in naturally contaminated foods. Competitive flora might inhibit *Y. enterocolitica* growth, even at refrigeration temperatures for several weeks, as in 41.9% of cases the microorganism could be isolated only from dilutions representing 0.1 and 0.01 g of the samples but not from tubes inoculated with 1.0 g of the samples.

The proposed cold-enrichment MPN method could be improved by using selective media other than CIN agar and primarily by testing enrichment broths by a PCR assay for *Y. enterocolitica* chromosome- and plasmid-borne genes. Target sequences could be the *ail* and the *virF* genes, as proposed by Hudson et al. *(41)*. Although CIN agar selective medium is recognized as the most acceptable agar for clinical samples, because of the high confirmation rate of presumptive isolates *(4)* it may inhibit the growth of some strains of *Y. enterocolitica* of pathogenic bioserotype 3/O:3 *(35)*. On CIN agar, recognition of *Y. enterocolitica* colonies is often difficult, as the typical bull's eye morphology is not always clearly distinguishable and other mannitol-fermenting bacteria produce similar colonies *(41)*. Therefore, plating samples onto a second medium may increase detection, such as, for example, SSI Enteric medium, which is effective in detecting *Y. enterocolitica (12),* or MacConkey agar, which is the most widely used *(31)*. A method based on PCR detection of some genes, such as *ail* and *virF,* in PSB dilution tubes after 24 to 48 h of cold enrichment could be reliable and time saving. PCR-positive enrichment tubes should be confirmed by isolation of *Y. enterocolitica* colonies on selective media. As observed in other studies, effective isolation media are needed, because isolation of *Y. enterocolitica* strains is essential for bacterial characterization and epidemiological information *(31)*.

In this study, the great majority of *Y. enterocolitica* isolates belonged to biotype 1A. The predominant genotype was *ystB*$^+$ (lacking *yadA, ail, inv,* and *ystA*), thus in agreement with other studies *(38, 58, 62, 67, 69)*. The *ystB* gene is considered a chromosomal marker strongly related to the biotype 1A *(69),* which encodes a heat-stable toxin similar to the one encoded by *ystA,* present both in nonpathogenic and noninvasive biotype 1A strains *(62)* and in partially pathogenic isolates *(63)*. In biotype 1A isolates the *inv* sequence was commonly detected. As the invasin protein induces proinflammatory host cell responses in human epithelial cells *(60), Y. enterocolitica inv*$^+$ isolates might be related to pathogenicity in the host. Although the presence of pYV in *Y. enterocolitica* biotype 1A is uncommon *(38),* it was recently observed in human clinical isolates of biotype 1A in China *(71)*. The genomic traits of the *Y. enterocolitica* 1A/O:7,8-8-8,19 strain (*yadA*$^+$ *inv*$^+$ *ystB*$^+$), detected in poultry meat, suggested that some biotype 1A isolates might be able to invade the epithelial cells *(38, 39)* and to avoid both phagocytosis by human polymorphonuclear cells and bacterial destruction by complement *(19, 20)*. As hypothesized in other studies and suggested by epidemiological surveys from human clinical samples, some biogroup 1A strains may be host adapted and potentially pathogenic to humans *(18, 48, 61)*.

Two *Y. enterocolitica* 2/O:9 strains were *ail*$^+$ *ystA*$^+$ (lacking *yadA, inv,* and *ystB*). The *ail* gene, located in the chromosome of pathogenic strains of *Y. enterocolitica,* is the most frequent genomic virulence marker, thus avoiding false-negative results due to pYV loss *(7, 16, 26, 68, 70)*. The presence of the *ystA* gene is supposed to be another important marker for pathogenicity among *Y. enterocolitica* isolates. As the pathogenic *Y. enterocolitica* strains carry the *ystA* gene and lack the *ystB* gene, testing for these two sequences could correctly identify most of the *Y. enterocolitica* isolates belonging to biotypes 1B, 2, 3, and 4 *(69)*. According to our data, the *ystA* gene was detected in eight isolates, five of them belonging to biotype 1A and three to biotype 2. Three *ystA*$^+$ biotype 1A strains of poultry origin carried also the *inv* and the *ystB* genes, thus suggesting a potentially pathogenic nature for these isolates.

Sensitivity of *Y. enterocolitica* to cefotaxime, ciprofloxacin, chloramphenicol, nalidixic acid, streptomycin, sulfonamide, tetracycline, trimethoprim, and sulfamethoxazole-trimethoprim is widely observed *(2, 6, 9, 43, 47)*. In contrast, resistance to gentamicin, an antibiotic considered to be 100% effective against *Y. enterocolitica (6, 36, 57),* or to aztreonam, a monobactam active toward *Y. enterocolitica* at very low MICs (0.03 to 0.25 mg/liter) *(66),* is uncommon.

Resistance of *Y. enterocolitica* to ampicillin is frequently reported *(1, 9, 25)*. Resistance to ampicillin and amoxicillin–clavulanic acid is due to the wide distribution of β-lactamases among *Y. enterocolitica* isolates *(17, 56)*. According to our data, in 93.3% of the isolates resistance to amoxicillin–clavulanic acid was linked to resistance to ampicillin. The difference in amoxicillin–clavulanic acid resistance rates between strains of pork (40.9%) and chicken (18.8%) origin had no statistical significance. Resistance to erythromycin was in accordance with previous investigations *(6, 34, 43, 57)*.

Since it is not known if antibiotic resistance in *Y. enterocolitica* is associated with plasmidic or chromosomal genes, it is important to test different antibiotics to study the resistance patterns of pYV$^+$ and pYV$^-$ isolates *(9)*. In our study, only one *Y. enterocolitica* 2/O:9 pYV-bearing isolate was resistant to erythromycin and to ampicillin. As a consequence, we could not suggest any association of the resistance profile with the presence or absence of the pYV.

The distribution of the virulence genes in the *Y. enterocolitica* isolates does not match any specific resistance pattern, even though we observed a higher resistance rate to amoxicillin–clavulanic acid in *ystB*$^+$ and in *inv*$^+$ *ystB*$^+$ strains.

Y. enterocolitica strains detected in foods are mostly biotype 1A, and isolates belonging to conventional pathogenic biotypes are rather uncommon if compared with their isolation rates from animal sources or from human clinical samples *(32, 37, 53, 61)*. Nevertheless, we suggest that even *Y. enterocolitica* biotype 1A isolates of food origin should be tested for virulence genes, because they might be potentially pathogenic to humans. Another relevant finding of this study is that chicken meat could be contaminated, like pork meat and products thereof, by *Y. enterocolitica* strains able to cause human infections.

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