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Variation in the Prevalence of Enteropathogenic *Yersinia* in Slaughter Pigs from Belgium, Italy, and Spain

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Abstract

Tonsils of 829 fattening pigs originating from Belgium ($n = 201$), Italy ($n = 428$), and Spain ($n = 200$) were collected between 2005 and 2007 to study the prevalence of enteropathogenic *Yersinia* in slaughter pigs. Isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* was done by selective enrichment and by cold enrichment for 7 and 14 days. Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* isolates were identified by polymerase chain reaction targeting the chromosomal genes *ail* and *inv*, respectively, as well as the plasmid-encoded *virF* of both species. A significantly higher ($p < 0.001$) prevalence of *ail*-positive *Y. enterocolitica* in Spain (93%) than in Belgium (44%) or Italy (32%) was observed. *virF*-positive *Y. enterocolitica* was present in 77% of *ail*-positive samples. Bioserotype 4/O:3 was the most common type in all three countries. Bioserotypes 2/O:5 and 3/O:9 were found in Italy (1%) and Belgium (9%), respectively. The prevalence of *inv*- and *virF*-positive *Y. pseudotuberculosis* was 2% and 1% in Belgium and Italy, respectively. *Y. pseudotuberculosis* was not detected in pigs from Spain. Bioserotypes 1/O:1 (20%), 1/O:2 (20%), and 2/O:3 (60%) were found in Belgium, and 1/O:1 (60%) and 2/O:3 (20%) in Italy. The most efficient method for isolation of *Y. enterocolitica* was combined cold enrichment for 7 and 14 days; however, the isolation method for *Y. pseudotuberculosis* was cold enrichment for 14 days. Fattening pigs seem to be an important reservoir of pathogenic *Y. enterocolitica* in Belgium, Italy, and Spain. Bioserotype 4/O:3 of *Y. enterocolitica* and bioserotypes 2/O:3 and 1/O:1 of *Y. pseudotuberculosis* have been shown to predominate.

Introduction

In Europe, sporadic yersiniosis cases related to *Yersinia enterocolitica* in humans are common, whereas outbreaks are rare (Fredriksson-Ahomaa *et al.*, 2009). However, large foodborne outbreaks due to *Yersinia pseudotuberculosis* have been reported in Finland and Russia, and also an increasing number of *Y. pseudotuberculosis* infections have been observed in France (Jalava *et al.*, 2004, 2006; Nuorti *et al.*, 2004; Anonymous, 2005, 2006; EFSA, 2006; Vincent *et al.*, 2008; Rimhanen-Finne *et al.*, 2009). In Belgium, Italy, and Spain, notification of yersiniosis is not compulsory; thus, no true incidence rates are available from these countries (ECDC, 2009).

Infections due to *Y. enterocolitica* and *Y. pseudotuberculosis* result in similar manifestations. Diarrhea is a common disorder among young children. Vomiting, fever, and abdominal pain are also symptoms that occur in yersiniosis patients (Anonymous, 2009a). Enteral yersiniosis in adults often goes unnoticed because of mild symptoms (Fredriksson-Ahomaa *et al.*, 2009; Rastawicki *et al.*, 2009). Reactive arthritis, erythema nodosum, and uveitis are examples of yersiniosis post-infectious sequels (Smego *et al.*, 1999; Fredriksson-Ahomaa *et al.*, 2009). Most human infections are due to *Y. enterocolitica* of bioserotype 4/O:3 (Anonymous, 2009a). All enteropathogenic *Yersinia* strains carry several essential chromosomal virulence genes such as *ail* and *inv*. A virulence plasmid (pYV) is needed for full pathogenicity; however, it can be easily lost during culturing (Bottone, 1999).

Contaminated pork is a suspected source of human yersiniosis cases (Kanazawa *et al.*, 1974; Tauxe *et al.*, 1987; Fredriksson-Ahomaa *et al.*, 2006; Grahek-Ogden *et al.*, 2007; Fosse *et al.*, 2008). Pigs are an important reservoir of *Y. enterocolitica*, especially bioserotype 4/O:3, which has a worldwide distribution. They frequently carry this pathogen in tonsils at slaughter (Fredriksson-Ahomaa *et al.*, 2006; Ortiz Martínez *et al.*, 2009). *Y. pseudotuberculosis* has sporadically been isolated from several animal species (Fredriksson-Ahomaa *et al.*, 2009). One potential reservoir for *Y. pseudotuberculosis* of bioserotype 2/O:3 is slaughter pigs (Kanazawa *et al.*, 1974;

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Niskanen *et al*., 2002, 2008; Ortiz Martínez *et al*., 2009). During the slaughtering of pigs, contamination of pluck sets (tongue, tonsils, and trachea hanging together with thoracic organs such as lungs, liver, and heart) and carcasses with enteropathogenic *Yersinia* from tonsils and feces may occur (Fredriksson-Ahomaa *et al*., 2001a, 2001b, 2009; Laukkanen *et al*., 2008, 2009b). Pork is the most consumed meat in Europe (Foreign Agricultural Service, U.S. Department of Agriculture, 2009). Pork and pork products are widely exported from Spain, Belgium, and Italy to other European countries (Anonymous, 2007, 2008, 2009c). Consumption of raw, undercooked, or improperly handled pork contaminated with *Y. enterocolitica* or *Y. pseudotuberculosis* may result in human infection (Kanazawa *et al*., 1974; Tauxe *et al*., 1987; Fredriksson-Ahomaa *et al*., 2006; Grahek-Ogden *et al*., 2007; Fosse *et al*., 2008). In Spain, pig meat, which was inadequately heat treated, was recently implicated in a small household *Y. enterocolitica* outbreak (Anonymous, 2009b).

Although pigs are considered a reservoir of enteropathogenic *Yersinia*, information about the prevalence and bioserotype distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* is limited in most European countries. This study was carried out to gain knowledge of the prevalence and distribution of different bioserotypes of enteropathogenic *Yersinia* in pigs in Belgium, Italy, and Spain by using selective and cold enrichments.

Materials and Methods

Sampling

Tonsils of 829 fattening pigs were collected between 2005 and 2007 at slaughter in the northern part of Belgium ($n = 201$), northern Italy ($n = 428$), and south-east Spain ($n = 200$). The pigs originated from 10 farms in Belgium, 22 farms in Italy, and 14 farms in Spain. Farms examined complied with the current EU-legislative requirements (EEC-Regulations, No. 2001/93). The tonsils were removed immediately after evisceration and placed in sterile sampling bags, frozen within 1–2 h after collection, and stored at $-20°C$ until examination.

Isolation of Y. enterocolitica *and* Y. pseudotuberculosis

Enteropathogenic *Yersinia* was isolated using selective enrichment according to ISO 10273:2003 (Anonymous, 2003), 7 days cold enrichment and 14 days cold enrichment as described by Niskanen *et al*. (2002) and Korte *et al*. (2004). A 10-g portion of tonsil tissue was homogenized in 90 mL phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts (PMB). For cold enrichment, the PMB was stored at 4°C for 7 and 14 days. The 14 days enrichment was followed immediately by an alkali treatment in 0.25% KOH solution for 20 sec before plating on a selective agar plate. For selective enrichment, 1 mL of the tonsil homogenate was transferred into 9 mL of irgasan–ticarcillin–potassium chlorate broth (Merck, Darmstadt, Germany) and incubated for 2 days at 25°C. Culturing on cefsulodin–irgasan–novobiocin (CIN) agar (Oxoid, Basingstoke, UK) was performed after every enrichment step, and CIN agar plates were incubated at 30°C for 24 h. Further, the plates were kept at room temperature for 2 days and checked for typical colonies (approximately 1.5 mm diameter with a dark pink center surrounded by a round pink and a translucent area). A maximum of three typical colonies on the CIN agar was streaked onto tryptic soy agar (Difco, Detroit, MI). Urease activity of the isolates was tested using an urea agar slant (Oxoid) incubated at 30°C for 24 h. Urease-positive isolates were biochemically identified with API 20E (BioMérieux, Marcy l'Etoile, France) incubated at 25°C for 18–20 h.

Biotyping and serotyping

Y. enterocolitica was biotyped based on its ability to ferment sugars (xylose, trehalose, salicine), pyrazinamidase activity, and tween and esculin hydrolysis (Wauters *et al*., 1987). The biotype of *Y. pseudotuberculosis* was determined as described by Tsubokura and Aleksić (1995) based on citrate utilization and melibiose and rhamnose fermentation. Serotyping was done by a slide agglutination test with commercial antisera O:1-O:3, O:5, and O:9 (Denka Seiken, Tokyo, Japan) for *Y. enterocolitica*, and antisera O:1-O:6 for *Y. pseudotuberculosis* (Denka Seiken).

Confirmation of pathogenic Y. enterocolitica *and* Y. pseudotuberculosis *by polymerase chain reaction*

Y. enterocolitica and *Y. pseudotuberculosis* isolates were further confirmed by polymerase chain reaction targeting the chromosomal genes *ail* (*Y. enterocolitica*) and *inv* (*Y. pseudotuberculosis*) according to Nakajima *et al*. (1992). Further, the presence of the virulence plasmid was shown by determining *virF* of *Y. enterocolitica* and *Y. pseudotuberculosis* according to Nakajima *et al*. (1992).

Statistical analysis

A 95% confidence interval for the prevalence of pigs positive for *Y. enterocolitica* and *Y. pseudotuberculosis* in three countries was calculated taking into account the number of pigs coming from the same farm (clustering) (Laukkanen *et al*., 2008) using the EpiInfo 6 program (Dean *et al*., 1994). When clustering, the design effect (impact of sampling in clusters) was calculated first, and then using the design effect the confidence interval was calculated with the Fleiss quadratic method using EpiInfo 6 program. Differences in the prevalence of pig farms positive for *Y. enterocolitica* and *Y. pseudotuberculosis* between Belgium, Italy, and Spain were compared with the nonparametric Mann–Whitney *U*-test (from the highest to the lowest *Y. enterocolitica* or *Y. pseudotuberculosis* farm prevalence) using Statistical Package for Social Sciences (SPSS, Chicago, IL). In addition, the McNemar test for dependent samples was used to compare the ability of different methods to detect *Y. enterocolitica* and *Y. pseudotuberculosis* using SPSS.

Results

Prevalence of ail*-positive* Y. enterocolitica *and* inv*-positive* Y. pseudotuberculosis

A significantly higher ($p < 0.001$) prevalence of *ail*-positive *Y. enterocolitica* in Spain (93%) than in Belgium (44%) or Italy (32%) was observed (Table 1). No statistically significant difference in prevalence between pigs from Belgium and Italy emerged. *Ail*-positive *Y. enterocolitica* was isolated from pigs originating from all farms studied in Italy (22) and Spain (14), whereas pigs from 2 of 10 farms were negative in Belgium.

The prevalence of *inv*-positive *Y. pseudotuberculosis* was 2%, 1%, and 0% in pigs from Belgium, Italy, and Spain, respectively (Table 1). *Y. pseudotuberculosis* was not found on Spanish farms, although 80% and 14% of farms in Belgium and Italy were positive, respectively. However, differences in prevalence between pigs from Belgium, Italy, and Spain were not significant ($p > 0.05$).

Altogether 77% (317/411) of *ail*-positive *Y. enterocolitica* samples were *virF* positive. All *Y. pseudotuberculosis* isolates were *virF* positive. *Y. enterocolitica* and *Y. pseudotuberculosis* were both isolated from one pig from Belgium (0.5%).

Isolation of enteropathogenic Yersinia *depending on the enrichment method*

Y. enterocolitica was isolated from 35% and 45% of the pigs by using selective enrichment and cold enrichment for 7 and 14 days, respectively, and in 50% of the samples when the results of all methods were combined (Table 2).

The combination of cold enrichment after 7 and 14 days was significantly ($p < 0.001$) more productive than selective enrichment for the isolation of *Y. enterocolitica* from pig samples (McNemar test) (Table 2). *Y. pseudotuberculosis*-positive samples were not detected after selective enrichment. Cold enrichment after 14 days yielded a significantly ($p < 0.05$) higher isolation rate of *Y. pseudotuberculosis* than cold enrichment after 7 days (McNemar test).

Bioserotypes of enteropathogenic Yersinia

Y. enterocolitica 4/O:3 was the predominant bioserotype recovered among Belgian (91%), Italian (99%), and Spanish (100%) positive pig samples (Table 3). Bioserotypes 3/O:9 and 2/O:5 were only observed among Belgian (9%) and Italian pigs (1%), respectively.

Three different bioserotypes of *Y. pseudotuberculosis*—1/O:1, 1/O:2, and 2/O:3—were present among pigs from Belgium, being *Y. pseudotuberculosis* 2/O:3 the most common (Table 3). In Italy, two bioserotypes of *Y. pseudotuberculosis*—1/O:1 and 2/O:3—were observed, with *Y. pseudotuberculosis* 1/O:1 predominating.

Discussion

Pathogenic *Y. enterocolitica* 4/O:3 was commonly present in pigs from Belgium, Italy, and Spain. The prevalence of *ail*-positive *Y. enterocolitica* isolated from the tonsils of fattening pigs was extremely high (93%) in Spain, indicating that this pathogen is very common in southern Europe. A high prevalence (89%) among pigs has also been reported in Estonia (Ortiz Martínez *et al*., 2009). Slightly lower isolation rates have been described for Latvia (64%), Germany (38–60%), Finland (36–37%), Russia (34%), and Switzerland (34%) (Asplund *et al*., 1990; Fredriksson-Ahomaa *et al*., 2000, 2001a; Gürtler *et al*., 2005; Fredriksson-Ahomaa *et al*., 2007; Ortiz Martínez *et al*., 2009). The lowest prevalence of the bioserotypes of *Y. enterocolitica* associated with human disease in fattening pigs has been reported in Poland (4%), Greece (13%), and Italy (15%) (Bonardi *et al*., 2003; Kechagia *et al*., 2007; Kot *et al*., 2007). However, our study shows that the prevalence of pathogenic *Y. enterocolitica* is high also in southern European countries. The low prevalence reported earlier in Italy (Bonardi *et al*., 2003) could be due to the isolation method used.

Y. pseudotuberculosis was isolated from pig tonsils from Belgium and Italy. All *inv*-positive *Y. pseudotuberculosis* recovered contained the virulence plasmid. The prevalence of *Y. pseudotuberculosis* has been at similar levels on pig farms in Estonia (1%), Finland (4%), Italy (0.3%), Germany (6%), Latvia (5%), Russia (7%), and the Netherlands (4%) (Narucka and Westendoorp, 1977; Weber and Knapp, 1981; Chiesa *et al*., 1993; Niskanen *et al*., 2002; Ortiz Martínez *et al*., 2009).

Selective enrichment yielded a lower recovery of *Y. enterocolitica* and *Y. pseudotuberculosis* among pig samples than either the combination of 7 and 14 days of cold enrichment or cold enrichment for 14 days. Cold enrichment has increased the number of *Y. enterocolitica* and *Y. pseudotuberculosis* isolates recovered from pigs in previous studies (Niskanen *et al*., 2002; Laukkanen *et al*., 2009a; Ortiz Martínez *et al*., 2009). Low selectivity of PMB together with cold enrichment allows the growth of both enteropathogenic *Yersinia* spp., with competitive microflora reduced by using KOH treatment.

Bioserotype 4/O:3 of *Y. enterocolitica* was predominant, as previously observed in slaughtered pigs from other European countries, including Denmark, Estonia, Greece, Italy, Finland, Germany, Latvia, Norway, Russia, Sweden, Switzerland, and Poland (Nielsen and Wegener, 1997; Fredriksson-Ahomaa *et al*., 2000, 2007; Bonardi *et al*., 2003; Gürtler *et al*., 2005; Platt-Samoraj *et al*., 2006; Kechagia *et al*., 2007; Kot *et al*., 2007; Ortiz Martínez *et al*., 2009). Two less common bioserotypes (3/O:9 and 2/O:5) associated with human disease were also found. *Y. enterocolitica* 3/O:9 (2%) was present among pigs in Belgium. Bioserotype 3/O:9 is not commonly found among European pigs; it has only sporadically been isolated from pigs in Great Britain (6%) (Milnes *et al*., 2008). Serotype O:9 of *Y. enterocolitica* has sporadically been isolated from German (0.3%) and Italian pigs (4%) (Bonardi *et al*., 2003; Gürtler *et al*., 2005). *Y. enterocolitica* 3/O:5,27 was the most common bioserotype isolated among pigs in Great Britain (Milnes *et al*., 2008). In this study, bioserotype 2/O:5 was present in Italian pigs. The same type has been isolated from 3% of Swiss pigs (Fredriksson-Ahomaa *et al*., 2007).

In Belgium, serotypes O:3 and O:9 have been shown to predominate in human yersiniosis (Verhaegen *et al*., 1998). Our study shows that Belgian pigs carry bioserotypes 4/O:3 and 3/O:9 in tonsils. Bioserotypes 4/O:3 and 2/O:9 have earlier been recovered among Italian children (Mingrone *et al*., 1987). However, in our study, pigs were a reservoir for bioserotypes 4/O:3 and 2/O:5 in Italy. *Y. enterocolitica* bioserotype 4/O:3 has been the only type reported among human yersiniosis cases in different Spanish regions such as Asturias, Barcelona, Guipuzcoa, and Madrid (Gurgui *et al*., 1988; Pérez-Trallero *et al*., 1992; Gómez-Garcés *et al*., 1996; Lobato *et al*., 1998). Bioserotype 4/O:3 was also the only type found in Spanish slaughter pigs in this study.

Y. pseudotuberculosis bioserotype 2/O:3 has thus far been the only bioserotype isolated from tonsils of pigs in Estonia, Finland, Latvia, and Russia (Niskanen *et al*., 2002, 2008; Ortiz Martínez *et al*., 2009). Serotype O:3 of *Y. pseudotuberculosis* has also been isolated from healthy pigs in Japan (Shiozawa *et al*., 1988). *Y. pseudotuberculosis* O:1 has sporadically been isolated from tonsils of German pigs (Weber and Knapp, 1981) and seemed to be the main serotype among human yersiniosis cases reported in France (Vincent *et al*., 2008; Rimhanen-Finne *et al*., 2009). In Finland, both serotypes O:1 and O:3 have been common in outbreaks (Jalava *et al*., 2004, 2006; Nuorti *et al*.,

Table 1. Prevalence of *ail*-Positive *Yersinia enterocolitica* and *inv*-Positive *Yersinia pseudotuberculosis* in Tonsils of Fattening Pigs from Belgium, Italy, and Spain

		Pigs							*Farms*						
		Yersinia enterocolitica			Yersinia pseudotuberculosis				Yersinia enterocolitica			Yersinia pseudotuberculosis		
Country	*No. of pigs*	*No. of positives*	*Prevalence (%)*	*95% Confidence interval*[a]	*No. of positives*	*Prevalence (%)*	*95% Confidence interval*[a]	*No. of farms*	*No. of positives*	*Prevalence (%)*	*95% Confidence interval*[b]	*No. of positives*	*Prevalence (%)*	*95% Confidence interval*[b]
Belgium	201	89	44	22–69	5	2	1–8	10	8	80	44–98	8	80	44–98
Italy	428	137	32	25–40	5	1	0.5–2.7	22	22	100	85–100	3	14	3–35
Spain	200	185	93	81–98	0	0	0	14	14	100	77–100	0	0	0
Total	829	411	50	39–60	10	1	0.6–2	46	44	96	85–100	11	24	13–39

[a]95% Confidence interval taking into account that 20–21 pigs from Belgium, 12–48 pigs from Italy, and 8–19 pigs from Spain originated from the same farm. The design effect (impact of sampling in clusters) was calculated, and then using the design effect the confidence interval was calculated with the Fleiss quadratic method.
[b]95% Confidence interval was calculated by exact binomial estimates.

Table 2. Recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from Pig Tonsils Depending on the Enrichment Method

			Cold enrichment							
	Selective enrichment[a]		*7 days*[c]		*14 days + KOH*[d]		*Total*[e]		*Total*[b]	
Species	*No. of positive tonsils*	*%*	*No. of positive tonsils*	*%*	*No. of positive tonsils*	*%*	*No. of positive tonsils*	*%*	*No. of positive tonsils*	*%*
Yersinia enterocolitica	288	35	302	36	307	37	372	45	411	50
Yersinia pseudotuberculosis	0	0	4	0.5	10	1	10	1	10	1

[a]Selective enrichment in irgasan-ticarcillin-potassium chlorate broth (ITC) according to Anonymous (2003) (ISO 10273:2003) and plating on CIN.
[b]Combined total for all enrichments: selective enrichment and cold enrichment for 7 and 14 days.
[c]Cold enrichment in phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts (PMB) and plating on CIN according to Korte *et al.* (2004) and Niskanen *et al.* (2002).
[d]Cold enrichment in PMB with KOH treatment and plating on CIN according to Korte *et al.* (2004) and Niskanen *et al.* (2002).
[e]Combined total for both cold enrichments: 7 and 14 days.
CIN, cefsulodin–irgasan–novobiocin; PMB, phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts.

Table 3. Bioserotypes of *ail*-Positive *Yersinia enterocolitica* and *inv*-Positive *Yersinia pseudotuberculosis* Pig Tonsils

	Yersinia enterocolitica							Yersinia pseudotuberculosis								
		2/O:5		*3/O:9*		*4/O:3*			*1/O:1*		*1/O:2*		*2/O:3*		*2/NT*[c]	
Country	n[a]	*No. of positives*	*%*	*No. of positives*	*%*	*No. of positives*	*%*	n[b]	*No. of positives*	*%*	*No. of positives*	*%*	*No. of positives*	*%*	*No. of positives*	*%*
Belgium	89	0	0	8	9	81	91	5	1	20	1	20	3	60	0	0
Italy	137	1	1	0	0	136	99	5	3	60	0	0	1	20	1	20
Spain	185	0	0	0	0	185	100	0	0	0	0	0	0	0	0	0
Total	411	1	0.2	8	2	402	98	10	4	40	1	10	4	40	1	10

[a]*n*: number of *ail*-positive *Y. enterocolitica* pig tonsils.
[b]*n*: number of *inv*-positive *Y. pseudotuberculosis* pig tonsils.
[c]NT, nontypeable isolates by serotyping.

2004). To reduce the potential transmission of enteropathogenic *Yersinia* from pork to humans, preventive measures starting at the pig farms and followed by the hygienic handling of pork meat during slaughter, in processing plants, and at points of consumptions are needed (Kapperud, 1991; Laukkanen *et al*., 2008).

In conclusion, human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* were commonly found among slaughter pigs from Belgium, Italy, and Spain and represent a potential food safety risk for humans in these countries. *Y. enterocolitica* prevalence was extremely high in Spain relative to Belgium and Italy. Further, *Y. enterocolitica* 4/O:3 among all countries studied and *Y. pseudotuberculosis* 1/O:1 and 2/O:3 in Belgium and Italy were the main bioserotypes isolated from pig tonsils. Cold enrichment was more effective in isolating both enteropathogenic *Yersinia* spp. than selective enrichment.

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Disclosure Statement

No competing financial interests exist.

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