Detection of pathogenic *Yersinia enterocolitica* in slaughtered pigs by cultural methods and real-time polymerase chain reaction

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Abstract

Healthy pigs carrying pathogenic to human *Yersinia enterocolitica* strains are the main source of entry into slaughterhouse, where cross-contamination of carcasses can happen. The aim of this work was to determine *Y. enterocolitica* prevalence in slaughtered pigs, investigating the presence of carriers in relation to carcass contamination. A total of 132 pig samples (tonsils, mesenteric lymph nodes, colon content, carcass surface) were collected from 4 Sardinian slaughterhouses. All the samples were examined by the ISO 10273:2003 method, and the prevalence was also determined by direct plating on CIN Agar. Moreover, to detect the *ail* positive *Y. enterocolitica* strains in enrichment broths and isolates a real-time polymerase chain reaction (PCR) was applied. *Y. enterocolitica* prevalence was 19% with direct plating and 12% with enrichment methods. Carcass surfaces and tonsils prevalence was 5.30% by direct plating, and 5.3% and 2.2%, respectively, by enrichment method. Tonsil samples showed an average contamination level of 3.2×10^3 CFU/g, while the mean value on carcass was 8.7×10^2 CFU/g. An overall prevalence of 9.8% of *ail* positive *Y. enterocolitica* broths was detected by RT-PCR, that found a higher prevalence in tonsils (7.5%) with respect to cultural methods, confirming the greater sensitivity of this technique when applied for tonsils and faeces samples. The results show a relatively low pathogenic *Y. enterocolitica* prevalence in pigs slaughtered in Sardinia. Good hygiene measures should be applied at slaughterhouse in order to prevent the entry of carriers and control carcass contamination.

Introduction

Yersinia is a psychrotrophic bacterium which belongs to *Enterobacteriaceae* widely distributed in natural environments (Kuan Tan *et al.*, 2014). The bacterial genus *Yersinia* comprises three main species that can be responsible of human infections: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*. Yersiniosis caused by *Y. enterocolitica* occurs mostly in young children causing diarrhea, sometimes bloody. Furthermore, in old people and patients with concomitant pathologies (iron overload, cirrhosis, diabetes, cancer, *etc*.), systemic forms of the disease are often observed (EFSA, 2013). *Y.enterocolitica* has 60 serogroups but the serotypes O:3, O:5, 27, O:8 and O:9 are the most frequently isolated from human clinical cases (Bolton *et al.*, 2013).

Y. enterocolitica is one of the most important foodborne enteric pathogen in Europe (Kuan Than *et al.*, 2014). The confirmed human cases of yersiniosis in the European Union in 2011 were 7017, and the most part was caused by *Y. enterocolitica* (98.4% of the confirmed cases), followed by *Y. pseudotuberculosis* (0.9%) (EFSA, 2013).

Slaughtered pigs are considered the principal animal reservoir for pathogenic strains of *Y. enterocolitica* (Van Damme *et al.*, 2013a), especially bioserotype 4/O:3, and human infection occurs primarily through the consumption of raw or undercooked pork meat or by direct contact with contaminated carcasses (Fondrevez *et al.*, 2014). *Y. enterocolitica* is often detected in tonsils, intestines and lymph nodes of swine and may spread and contaminate the carcass surface during slaughtering (Van Damme *et al.*, 2013a). Furthermore, this microorganism is able to grow at refrigeration temperatures and to survive in frozen pork meat, and grows well under modified atmosphere conditions (Martinez, 2010).

Pathogenicity of *Y. enterocolitica* depends on chromosomally encoded virulence factors. The *ail* gene, provides resistance to killing by human serum, the *yst* (*ystA* and *ystB*) genes encode for a enterotoxin responsible of causing diarrhoea during infection; finally, the *inv* gene is required for the invasion of the intestinal epithelium of the host (Bolton *et al.*, 2013).

The International Standard Organization method (ISO10273; ISO, 2003) usually applied for *Y. enterocolitica* detection in food samples and pig tonsils, has been shown to be ineffective for isolation of *Y. enterocolitica* in food with low contamination levels (Van Damme *et al.*, 2010, 2013b). In particular, the huge growth of competing flora can make difficult to isolate *Y. enterocolitica* colonies on selective medium, causing false negative results (Van Damme *et al.*, 2013b).

Polymerase chain reaction methods can significantly improve the detection of *Y. enterocolitica* in food. The real-time polymerase chain reaction (PCR) procedures were in particular developed to detect the *ail* gene, considered an excellent target to found the pathogenic *Yersinia* strains, providing greater specificity respect to conventional PCRs (Thisted Lambertz *et al.*, 2008). Boyapalle *et al.* (2001) reported that real-time PCR assay is 1000 to 10,000 times more sensitive than the culture methods or the traditional PCR assay when tonsil and faecal samples are investigated (Fredriksson-Ahomaa *et al.*, 2007).

The aim of this work was to determine *Y. enterocolitica* prevalence in slaughtered pigs by the (ISO) cultural (modified) and the real-time PCR methods. Moreover, the virulence profile of the isolates was determined by PCR methods, in order to evaluate the potential risk to human health.

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Key words: *Yersinia enterocolitica*; Slaughtered pig; Carcass; *Ail* gene; Real-time PCR.

Conflict of interest: the authors declare no potential conflict of interest.

Received for publication: 16 July 2014.
Revision received: 9 December 2014.
Accepted for publication: 9 December 2014.

Italian Journal of Food Safety 2015; 4:4579
doi:10.4081/ijfs.2015.4579

Materials and Methods

A total of n. 132 samples collected from 33 finishing pigs, taken from 4 Sardinian slaughterhouses (A, B, C, D), were examined for *Y.enterocolitica* presence.

Samples collection

Samples from pig tonsils (25%), mesenteric lymph nodes (25%), colon content (25%) and carcass surface (25%) were collected, as previously described (Piras *et al.*, 2011).

Detection and enumeration of *Yersinia enterocolitica*

Detection and enumeration of *Y. enterocolitica* were carried out by the ISO 10273-2003 protocol, modified as described by Van Damme *et al.* (2010, 2013b). All the samples were suspended in Yersinia PSB broth (Biolife, Milan, Italy) and homogenized in a Stomacher blender. After incubation for 2 h at room temperature, a 10 µL aliquot was streaked onto Cefsulodin-Irgasan-Novobiocin agar (CIN agar; Biolife) plates. Then, colonies with typical aspect (red color surrounded by translucent white zone) were enumerated and n. 5 of each sample streaked onto CIN agar plates. In parallel, all PSB broths were incubated at 25°C for 2 (PSB 2d) and 5 days (PSB 5d). Subsequently, 0.5 mL of the PSB broth were transferred to 4.5 mL of a 0.5% potassium hydroxide (KOH) solution and streaked in CIN agar plates (PSB KOH). At the same time, 10 µL of PSB broth, without alkali treatment, were streaked in CIN agar plates. All agar plates were incubated at 30°C for 24 h.

Phenotypic identification

All typical colonies were tested for oxidase and catalase enzymes, and by seeding in Kligler Iron Agar (KIA; Biolife), incubated at 30°C for 24 h. Moreover, presumptive colonies were submitted to phenotypic identification with API® 20 E system (bioMérieux, Marcy l'Etoile, France).

Yersinia enterocolitica detection and identification by molecular procedures

All the PSB broths, after incubation at 25°C for 2 days, without KOH treatment, and a subset of *Y. enterocolitica* strains isolated from CIN Agar, and previously confirmed by phenotypical identification, were subjected to molecular tests. The DNA extraction procedure used the chelation properties of Chelex resin. The real-time PCR test, carried out in order to identify *Y.enterocolitica* by *ail* detection, was performed as described by Thisted Lambertz *et al.* (2008) protocol, modified. The PCR conditions were the following: 1X TaqMan universal PCR master mix (containing AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates and optimized buffer components; Applied Biosystems, Foster City, CA, USA), primers (*ye-ail-F2* and *ye-ail-R2*) to a final concentration of 300 nM, and probe (*ye-ail-tmp*) to a final concentration of 125 nM. The heterologous Internal Amplification Control System based on the pUC 18 plasmid was applied with primers (pUC 18-F and pUC 18-R) to a final concentration of 250 nM and probe (IAC Tm-pUC 18) to a final concentration of 100 nM. 2.5 µL of the sample and 1 µL of the pUC 18 plasmid were added to 21.5 µL of primary mix. Sterile MilliQ water was used to adjust the volume of each reaction mixture to 25 µL. The PCR cycling parameters were as follows: initial denaturation of the template DNA at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and at 60°C for 30 s.

Results

In Table 1 the results of *Y. enterocolitica* detection by direct plating, PSB enrichment after 2d and 5d (with and without alkali treatment), and real-time PCR, performed on broth and on isolates, are showed in relation to the slaughterhouse and the sampling site.

The prevalence of *Y. enterocolitica* was 19% (n. 25) with direct plating, and 12% (n. 16) with enrichment methods. *Y. enterocolitica* prevalence differed between slaughterhouses: slaughterhouse A showed the highest prevalence (16%), followed by D (7%), B (6%) and C (4%). In detail, among the positive samples detected by direct plating, colon content showed a prevalence of 6.8% (n.9), carcass surface and tonsils 5.30% (n. 7), in and 1.5% (n.2) in mesenteric lymph nodes. The mean contamination level registered by direct plating was unhomogeneous, accounting for 34.800±60.996 (mean±SD) CFU/g. Positive tonsil samples showed a contamination level, ranging from 1.0×10^2 to 1.5×10^4 CFU/g, with an average of 3.2×10^3 CFU/g. Moreover, the level of contamination in carcass samples was variable, ranging from 1.0×10^2 CFU/g to 2.5×10^3 CFU/g, with a mean value of 8.7×10^2 CFU/g.

With enrichment methods, *Y. enterocolitica* was isolated from 5.3% of carcass surface (n. 7), 3.8% (n.5) of colon content, 2.2% (n. 3) of tonsils, and 0.8% (n. 1) of mesenteric lymph nodes, accounting for a total of 16 samples.

Within the positive samples detected by direct plating (Table 1), n. 3 samples (1 lymph node, 1 tonsil, 1 colon content) were also positive by PSB 2d, n. 3 (1 colon content, 1 carcass surface, 1 tonsil) by PSB+KOH 2d, n.2 (1 colon content, 1 tonsil) by PSB 5d, and n. 1 (colon content) by PSB+KOH 5d. All the samples were from different plants.

Among the n. 16 samples positive by enrichment procedures, n. 10 were detected after 2d and n.8 after 5d incubation. In particular: i) n. 5 were detected by PSB 2d (1 lymph node, 2 carcass surface, 1 colon content, 1 tonsil), and n. 5 by PSB KOH 2d (1 colon content, 3 carcass surface, 1 tonsil); ii) 7 by PSB 5d (2 colon content, 4 carcass surface, 1 tonsil), and n. 3 by PSB KOH 5d (1 colon content, 2 carcass surface). After 5d incubation in PSB, 2 samples of carcass surface resulted positive both with and without alkali treatment. Moreover, n. 2 different carcass surface samples, positive after 2d incubation in PSB, were confirmed after 5 d incubation.

Seven samples resulted positive by enrichment procedures and negative by direct plating and between these, only 2 were positive after both enrichment incubation periods. Between the 16 samples that were positive with the enrichment methods, 9 (4 colon content, 1 lymph node, 1 carcass surface, 3 tonsils) resulted positive to direct plating too.

Detection of *ail* virulence gene

In total, n. 13 (9.8%) *ail* positive broths, from lymph nodes (1, *Ct* value 39.2), carcass surfaces (2, C_t values between 35.6 and 37.0) and tonsils (10, C_t values between 20.5 and 36.9), were detected by real-time PCR. Within the *ail* positive broths, 3 tonsils resulted positive also to direct plating ($1.5x10^4$, $2.0x10^2$, and $1.0x10^2$ CFU/g), one of which were positive by PSB 2d and one by PSB KOH 2d. Ten samples (1 lymph node, 2 carcass surface, 7 tonsils), were detected positive only by real-time PCR. The *ail* positive samples were from the plant A (n. 3), plant C (n. 3), and plant D (n.7). On the contrary, none of the samples collected from plant B resulted positive by real-time PCR. No relation between C_t values and *Y.enterocolitica* contamination count by direct plating was showed.

Overall, the *ail* gene was detected by real-time PCR in n. 10/34 (29.4%) strains, isolated from carcass surfaces (3), colon content (3) and tonsils (4). Between these, 8 were isolated from PSB 2d, and 2 from PSB 5d. No differences between alkali treated and not alkali treated samples, were detected. Finally, 2 *ail* positive strains were detected with real-time PCR in samples whose broths resulted positive too with the same molecular method.

Discussion

Y. enterocolitica prevalence detected by cultural methods in tonsil samples were lower than those reported in previous surveys carried out in Italy, that showed prevalence comprised between 10.8 and 15.3% (Bonardi *et al.*, 2013, 2014), and other European countries (Fredriksson-Ahomaa *et al.*, 2001; Van Damme *et al.*, 2010). Our results reflect a low infection prevalence in the pig population slaughtered in Sardinia, in spite of the mean live weight (range 100-120 kg) and the age (7-8 months) of the pigs included in our survey (Gürtler *et al.*, 2005). The contamination level in tonsils resulted comparable with those detected in Italian (Bonardi *et al.*, 2013) and Belgian pigs (Van Damme *et al.*, 2010), representing a possible risk for carcasses and fresh pork meat. In this study the prevalence in carcass samples was lower (~ 1-2 log) than in tonsils.

The results of the detection of *ail*-positive *Y.*

Table 1. Results of *Yersina enterocolitica* detection by cultural methods (direct plating, 2 and 5 days enrichment broths) and real-time polymerase chain reaction.

Slaughterhouse	Source	Direct plating (cfu/g)	Enrichment broths incubation time				Real-time PCR	
			2 d		5 d			
			PSB	KOH	PSB	KOH	Broth	Isolates
A	Cc	1.0×10^{2}			+		-°	-
“[#]	“						-	
“	“						-	
A	Cc	1.2×10^{4}				+	-	-
A	Cc	1.0×10^{2}		+			-	-
A	Cc				+		-	-
“	“						-	
“	“						-	
A	Cc	2.8×10^{3}					-	-
A	Cc	2.3×10^{3}					-	-
A	Cc	1.8×10^{3}					-	-
A	Ln						+	-
A	Ln	8.0×10^{2}					-	-
A	Ln	2.0×10^{3}	+				-	-
A	Cs		+		+	+	-	-
“	“							+
“	“					-		
A	Cs				+	+	-	-
“	“							-
“	“							-
“	“							-
A	Cs			+			+	+
A	Cs		+		+		-	+
“	“							-
A	Cs	1.3×10^{3}		+			-	-
A	Cs				+		-	-
A	Cs			+			-	-
A	Cs	1.0×10^{2}					-	-
A	T[d]	1.7×10^{4}			+		-	-
“	“						-	
“	“						+	
“	“						-	
A	T	1.5×10^{4}	+				+	+
“	“						+	
“	“						+	
A	T	4.3×10^{3}					-	-
B	Cc	3.0×10^{3}					-	-
B	Cc	6.8×10^{3}					-	-
B	Cc	2.68×10^{3}	+				-	+
“	“						+	
“	“						+	
B	Cs	7.0×10^{2}					-	-
B	Cs	2.5×10^{3}					-	-
B	Cs	1.3×10^{3}					-	-
B	T	5.0×10^{2}					-	-
B	T	6.0×10^{2}					-	-
C	Cs						+	-
C	Cs	1.0×10^{2}					-	-
C	Cs	1.0×10^{2}					-	-
C	T	2.0×10^{2}		+			+	-
C	T						+	-
D	T						+	-
D	T						+	-
D	T						+	-
D	T						+	-
D	T						+	-
D	T						-	-
D	T						-	-
D	T	1.0×10^{2}					+	-
D	T						+	-

PCR, polymearse chain reaction; Cc, colon content; Ln, lymph node; Cs, carcass surface; T, tonsils. °*Ail* gene not detected; #strains isolated from the sample above XXX. Samples resulted positive for at least one method.

enterocolitica strains by real-time PCR showed a higher prevalence in tonsils (7.5%) than those detected by cultural methods (5.3 and 2.2 by direct plating and enrichments, respectively), confirming the greater sensitivity when applied for samples of tonsils and faeces. For example, Fredriksson-Ahomaa *et al.* (2007), by the comparison of cultural methods (direct plating, overnight enrichment and selective enrichment) with a real-time PCR to detect *ail*-positive *Y. enterocolitica* in pig tonsils collected in Switzerland, found a detection rate of 34 *vs* 88%, respectively (Fredriksson-Ahomaa *et al.,* 2007).

However, the isolation of *Y. enterocolitica* from food samples by at least one culture method is needed in order to acquire epidemiological information on human pathogenic bio-serotype circulation, and then it should be used in parallel to PCR method. The evaluation of the presence of the *ail* gene in the isolates is required to make a distinction from pathogenic and no-pathogenic *Y. enterocolitica* population. In this study, the *ail* gene was detected approximately in 30% of isolates, being not in agreement with other studies, where 87.5% of bio-serotype 4/O:3 and all of 2/O:9 were found *ail* positive (Bonardi *et al.*, 2013). These bio-serotypes were frequently isolated in human yersiniosis, while in other bio-serotyopes, as BT 1 A, rarely involved in human cases, the prevalence of *ail* gene may be lower. The typing of the strains will be carried out to characterize the pathogenic *Y. enterocolitica* isolated in our study.

Conclusions

The results of our survey show that the presence of pathogenic *Y. enterocolitica* strains in pigs slaughtered in Sardinia is relatively low. The contamination of carcass surfaces highlights that good hygiene measures are needed in order to control the spread at abattoir, *e.g.* during the evisceration practices and tonsils removal. Moreover, the further implementation of control programmes, aimed to reduce the presence of carrier pigs at farm level, will be the best strategy to prevent the contamination at slaughterhouse.

The direct plating allowed a quantitative estimation, and apparently showed a higher sensitivity with respect to enrichment procedures. However, because of the growth of a high number of natural background microorganisms in CIN agar plates, it is necessary to isolate a number of presumptive *Y. enterocolitica*, apparently similar to those pathogenic. The real-time PCR confirms to have a greater sensitivity respect to cultural methods for the detection of pathogenic *Y. enterocolitica* in animal and food samples. Further epidemiological findings can be acquired to better evaluate the results, by typing methods (bio-serotyping and pulsed field gel electrophoresis), in order to investigate the serotypes circulating in Sardinian pig population, their pathogenic profiles and to trace the routes of contamination at slaughterhouse.

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