The prevalence, characterisation, and antimicrobial resistance of Yersinia enterocolitica *in pigs from Central Italy*

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Veterinaria Italiana 2018, **54** (2), 115-123. doi: 10.12834/VetIt.1126.6109.2
Accepted: 16.10.2016 | Available on line: 30.06.2018

Keywords
Antimicrobials resistance, Bioserotype, PFGE, Virulence gene, *Yersinia enterocolitica*.

Summary
Widely spread in nature, *Yersinia enterocolitica* (YE) is a foodborne pathogen of major health and economic significance in developed countries. The aim of this study is to analyse YE strains isolated from 400 slaughtered pigs from the Abruzzo region, Italy, using biochemical tests and a multiplex polymerase chain reaction PCR detecting 6 chromosomal genes (*ystA, irp2, 16s, ail, inv, hemR*) and one plasmid-borne virulence gene (*yadA*). Antimicrobial susceptibility was evaluated and pulsed-field gel electrophoresis (PFGE) was also performed in order to assess phylogenetic diversity. In total, 56 samples of porcine tonsils (14%) were found to be positive for the presence of pathogenic YE. All YE belonged to the pathogenic bioserotype 4/O:3. All YE samples were positive for the chromosomal virulence genes *ystA, ail,* and *inv*, whereas results for the presence of *yadA* and *hem*R were variable. This study found that YE isolates were resistant to ampicillin (100%), streptomycin (26.79%), sulfisoxazole (19.65%), tetracycline (16.08%), nalidixic acid (14.30%), and chloramphenicol (10.72%). The strains characterised by PFGE showed a high similarity. This study demonstrates the usefulness of multiplex polymerase chain reaction (PCR) compared with conventional phenotypic assays for the identification of pathogenic YE isolates and the limitations of PFGE for the molecular typing of YE bioserotype 4/O:3.

Prevalenza, caratterizzazione e antibiotico resistenza di Yersinia enterocolitica *nei maiali del centro Italia*

Parole chiave
Antibiotico resistenza, Biosierotipo, PFGE, Geni di virulenza, *Yersinia enterocolitica*.

Riassunto
Yersinia enterocolitica (YE) è un enteropatogeno di origine alimentare ampiamente diffuso in natura e potenzialmente dannoso per il settore economico della sanità pubblica. Lo scopo di questo lavoro è stato quello di analizzare i ceppi di YE isolati da 400 suini al macello, provenienti da diversi allevamenti dell'Abruzzo, attraverso l'utilizzo di test biochimici e una PCR multiplex avente come target 6 geni cromosomici (*ystA, irp2, 16s, ail, inv, hemR*) e uno plasmidico (*yadA*). È stata inoltre valutata l'antibiotico resistenza dei ceppi ed è stata studiata la diversità genetica degli isolati con la elettroforesi in campo elettrico pulsato (PFGE). Nel complesso il 14% delle tonsille (56 campioni) sono risultate positive per la presenza di YE. Tutti i ceppi isolati sono risultati appartenere al biosierotipo 4/O:3. La PCR multiplex ha confermato l'identificazione biochimica dei ceppi e l'assenza dei biotipi 1A e 1B. Tutti gli isolati sono risultati positivi per i geni di virulenza *ystA, ail* e *inv*, mentre la presenza di *yadA* e *hemR* è risultata variabile. Gli isolati di YE sono risultati resistenti all'ampicillina (100%), streptomicina (26.79%), sulfisoxazolo (19.65%), tetraciclina (16.08%), acido nalidixico (4.30%), e cloramfenicolo (10.72%). La caratterizzazione mediante PFGE ha mostrato l'elevata similarità dei ceppi isolati. Questo studio dimostra che la PCR multiplex, rispetto ai test biochimici, è vantaggiosa nell'identificazione e nella caratterizzazione dei ceppi patogeni di *Yersinia enterocolitica* e che la PFGE ha limitazioni come metodica di caratterizzazione dei ceppi di biosierotipo 4/O:3.

Introduction

The bacterial genus *Yersinia* consists of 3 main species that are known to cause human infections: *Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*. Of these, *Yersinia enterocolitica* is the most commonly reported species in human cases, and infections are most often acquired by eating contaminated food, particularly raw or undercooked pork (EFSA and ECDC 2012).

The classification of *Y. enterocolitica* strains has traditionally been based on a combination of biochemical and serological tests into 6 bioserotypes. This species primarily comprises strains that are generally regarded as non-pathogenic and widely distributed in the environment (biotype 1A), strains of biotypes 2 -5, which are considered pathogenic, and strains considered highly pathogenic (biotype 1B) (EFSA and ECDC 2015).

Swine are the primary source for human pathogenic types of *Y. enterocolitica*, mainly biotype 4, serotype O:3 (bioserotype 4/O:3). The strains that are predominantly associated with human disease worldwide and in many European countries belong to bioserotype 4/O:3, with varying percentages in different countries (Falcao *et al.* 2006, Fredriksson-Ahomaa *et al.* 2001a, Korte *et al.* 2004, Martinez *et al.* 2011), followed by bioserotypes 1B/O:8, 2/O:9, and 2/O:5,27 (Bottone 1999, Drummond *et al.* 2012, EFSA 2007). Slaughtered pigs are recognised as the most important source for *Yersinia enterocolitica* (YE) foodborne transmission as they are the only food animals that commonly harbour these pathogenic bioserotypes (Andersen *et al.* 1991, Bucher *et al.* 2008, Fredriksson-Ahomaa *et al.* 2001b, Fredriksson-Ahomaa *et al.* 2006). Many studies demonstrate that porcine tonsils are the best way to detect *Y. enterocolitica* in slaughtered pigs (Gutler *et al.* 2005, Nesbakken *et al.* 2006, Thibodeau *et al.* 1999). Tonsils are also considered an important source for the contamination of pig offal and carcass (Fredriksson-Ahomaa *et al.* 2001 a, b). *Y. enterocolitica* bioserotype 4/O:3 can cause many gastrointestinal problems in humans. These vary from enterocolitis and pseudoappendicitis, to severe complications such as reactive arthritis or erithema nodosum (Lambertz *et al.* 2007). The YE virulence mechanism is still not clear, even if many chromosome- and plasmid-derived virulence factors involved in the pathogenicity and progression of yersiniosis have been studied and described (Paixao *et al.* 2013). The 70-kb plasmid, pYV, is a virulence factor in YE bioserotype 4/O:3. It confers several thermo-dependent properties, including the secretion of 12 proteins known as Yops (*Yersinia* outer proteins) and the production of 2 outer membrane proteins, YadA and YlpA (Cornelis *et al.* 1998, Robins-Browne 2007). YadA mediates bacterial adhesion, invasion, and resistance to immune responses (Drummond *et al.* 2012).

The highly pathogenic *Y. enterocolitica* biotype 1B also harbours the chromosomal high-pathogenicity island (HPI), which encodes proteins involved in the biosynthesis, regulation, and transport of the siderophore yersiniabactin. The 5 main genes within this island that are involved in the yersiniabactin system are *psn, irp1, irp2, ybtP,* and *ybtQ* (Carniel 2001, Rakin *et al.* 1999). Other genes most frequently used as a target for pathogenic isolates are *inv, ail, ystA*, and *hemR* (Revell and Miller 2001). The first 2 chromosomal genes are involved in the invasion of mammalian cells; the *inv* gene is present in both virulent and non-virulent strains of YE, while the *ail* gene is exclusively present in pathogenic isolates (Miller and Falkow 1988, Miller *et al.* 1989). The *ystA* gene encodes for a thermostable enterotoxin (Delor *et al.* 1990), while *hemR* gene encodes for a heme receptor that removes iron bound to heme proteins (Stojiljkovic and Hantke 1992).

It is well known that the expression of both plasmid and chromosomal genes is required for YE virulence. However, different studies have demonstrated that it is difficult to maintain the pYV plasmid during laboratory culturing, which increases the chances of obtaining a false-negative result when attempting to identify *Yersinia* isolates (Blais and Phillippe 1995, Thoerner *et al.* 2003, Zheng *et al.* 2008).

Until now, many molecular methods have been applied to investigate the genetic heterogenicity of YE. A great number of studies, especially those regarding YE bioserotype 4/O:3, demonstrate that these bacteria are well conserved and show a limited degree of genetic diversity (Falcao *et al.* 2006, Sachdeva and Virdi 2004). In northern Italy, recent studies in different abattoirs showed the presence of YE in 21% of pork origin isolates; among these, the 86% of YE resulted in YE bioserotype 4/O:3, and the most common virulence factors associated were *ystA, inv, ail*, and *yadA* (Bonardi *et al.* 2013, Bonardi *et al.* 2014). The aim of this study is to characterise pathogenic YE in slaughtered pigs located in central Italy using bioserotyping, antimicrobial susceptibility, multiplex PCR-detecting virulence factors, and pulsed-field gel electrophoresis (PFGE) methods.

The biotyping and characterisation of YE isolates in food animals is essential in order to determine the pathogenicity of different strains, as well as to investigate the sources, modes of transmission, and geographical distributions of isolates (Virdi and Sachdeva 2005). This information will, in turn, inform control measures in slaughtering procedures that can reduce the transmission of these pathogenic bacteria to pork meat.

Materials and methods

Sampling

From February - March 2011, 400 pigs slaughtered in 1 abattoir located in the Abruzzo region (central Italy) were tested for YE. The animals originated from 13 different pig farms spread across 2 provinces of Abruzzo at a distance of about 100 km each. Porcine tonsils were removed aseptically immediately after evisceration, placed into sterile plastic bags, and transported to the laboratory under chilled conditions, where they were tested immediately or within 24 hours of collection.

Culture collection strains

Table I shows YE reference strains and other reference bacteria used as positive or negative controls for biochemical and PCR tests.

Biotyping and serotyping

The samples were processed according to ISO 10273:2003 for the detection of YE, presumed to be pathogenic to human subjects. Presumptive YE strains were biochemically identified using a Vitek 2 Compact microbial identification system (bioMerieux SA, Marcy l'Etoile, France). *Y. enterocolitica* isolates were grouped by biotyping (Bottone 1999) with commercially available discriminatory tests for aesculin, xylose, trehalose, indole, as well as the Voges-Proskauer test (Liofilchem s.r.l, Roseto degli Abruzzi, Italy), and serotyped using the YE Antisera Set (Denka Seiken Co., Ltd., Tokyo, Japan).

DNA extraction, primers, and PCR conditions

DNA extraction was conducted using the Maxwell® 16 Cell DNA Purification Kit (Promega, Madison, WI, USA) in combination with a Maxwell® 16 Instrument (Promega). Extracted DNA was quantified using a spectrophotometer (NanoDrop Technologies, Celbio Srl., Milan, Italy). Primers used for the multiplex PCRs are listed in Table II. A primer set specific to the *hemR* gene, resulting in a 713-bp PCR product, was designed with Primer-BLAST (Ye *et al.* 2012). The specificity of the amplified sequence was tested by BLAST[1], searching the GenBank database. PCRs were performed in a 50 µL reaction mixture containing 5 µL of DNA template (≤ 1 µg per 50 µL), 25 µL of 2× Qiagen Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA), 5 µL of 10× primer Mix (containing 2 µmol L^{-1} of each primer), 5 µL of 5× Qiagen Q-Solution (Qiagen), and 10 µL of RNAse-free water. Polymerase chain reaction amplifications were performed with the GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) according to the Qiagen Multiplex PCR Master Mix kit cycling protocol. The samples were subjected to an initial

Table I. *Reference strains used for evaluation of the multiplex PCR assay.*

Species	Strain designation[a]	Biotype[b]	Serotype[b]
Y. enterocolitica	ATCC 51871	(1B)	0:8
Y. enterocolitica	ATCC 49937	(1B)	(0:8)
Y. enterocolitica	ATCC 27739	(1B)	(0:8)
Y. enterocolitica	ATCC 55075	(2)	0:9
Y. enterocolitica	ATCC 700823	2	0:9
Y. enterocolitica	ATCC 9610	1B	0:8
Y. enterocolitica	ATCC 23715	1B	0:8
Y. intermedia	NCTC 11469	-	-
Y. pseudotuberculosis	NCTC 10275	-	-
C. jejuni	ATCC 33291	-	-
E. coli	ATCC 25922	-	-
L. monocytogenes	ATCC 19111	-	-
S. typhimurium	ATCC14028	-	-
E. faecalis	ATCC 29212	-	-
P. aeruginosa	ATCC 27853	-	-
S. flexneri	ATCC 12022	-	-

[a] ATCC, American Type Culture Collection (LGC St&ards S.r.l, Milan, Italy); NCTC, National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK).
[b] The biotypes & serotypes in parentheses were typed in this study; the others were obtained from strain collection data.

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi

Table II. *Primers used for multiplex PCR assay.*

Primer	Amplicon size (bp)	Sequence (5'–3')	Reference
ystA	145	AATGCTGTCTTCATTTGGAGC	Ibrahim *et al.* 1997
		ATCCCAATCACTACTGACTTC	
irp2	264	AAGGATTCGCTGTTACCGGAC	Schubert *et al.* 1998
		TCGTCGGGCAGCGTTTCTTCT	
16s	330	AATACCGCATAACGTCTTCG	Neubauer *et al.* 2000
		CTTCTTCTGCGAGTAACGTC	
ail	454	GTTTATCAATTGCGTCTGTTAATGTGTACG	Thisted Lambertz *et al.* 2000
		CTATCGAGTTTGGAGTATTCATATGAAGCG	
inv	570	CTGTGGGGAGAGTGGGGAAGTTTGG	Rasmussen *et al.* 1994
		GAACTGCTTGAATCCCTGAAAACCG	
hemR	713	TTTCTGTTGCGCCGCTCGGT	This study, GenBank accession no. AM 286415.1
		TCCGGCGCTGGTTAAGCGTG	
yadA	849	CTTCAGATACTGGTGTCGCTGT	Thoerner *et al.* 2003
		ATGCCTGACTAGAGCGATATCC	

denaturation at 95 °C for 15 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 90 seconds, and 72 °C for 90 seconds, followed by a final primer extension at 72 °C for 30 minutes. The PCR products were analysed by capillary electrophoresis with a QIAxcel system (Qiagen), using a QIAxcel DNA Screening Cartridge (Qiagen).

Antimicrobial susceptibility

We evaluated YE strains susceptibility to antimicrobials with the microdilution method using the Sensititre automated system (TREK Diagnostic Systems, Cleveland, OH, USA), according to the recommendations of CLSI (CLSI 2012). We transferred 10 microliters of 0.5 McFarland bacterial suspension in Adjusted cation Mueller-Hinton Broth with TES, and dispensed it into CMV2AGNF microtiter plates (TREK Diagnostic Systems). The plates contained known scalar concentrations of the following antimicrobials: cefoxitin (0.5-32 µg/mL), chloramphenicol (2-32 µg mL), tetracycline (4-32 µg mL), ceftriaxone (0.25-64 µg/mL), amoxicillin/clavulanic acid (1/0.5-32/16 µg/mL), ciprofloxacin (0.015-2 µg mL), gentamicin (0.25-16 µg/mL), nalidixic acid (0.5-32 µg/mL), ceftiofur (0.12-8 µg/mL), sulfisoxazole (16-256 µg/mL), trimethoprim/sulfamethoxazole (0.12/2.38-4/76 µg/mL), kanamycin (8-64 µg/mL), ampicillin (1-32 µg/mL), and streptomycin (32-64 µg/mL). After inoculation, the plates were incubated at 37 °C in aerobic condition for 24 hours and then screened. We used *Escherichia coli* strain ATCC 25922 as a control.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was performed using 2 'rare cutting' enzymes: XbaI and NotI. Genomic DNA was prepared using the PulseNet protocol for *Y. pestis*, with some modifications (CDC 2011). Briefly, bacteria grown for 24 hours on tryptic soy agar were suspended in 2 mL of cell suspension buffer (100 mmol L^{-1} Tris:100 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), pH 8.0) (Lonza, Basel, Switzerland, and Life Technologies, Grand Island, NY, USA, respectively) to a cell concentration corresponding to 3 McFarland units (bioMerieux SA). Twenty microliters of proteinase K (20 mg mL^{-1}) (Sigma-Aldrich, Buchs, SG, Switzerland) were added to 400 µL of the cell suspension, and then mixed with an equal volume of 1% of melted Seakem Gold agarose (Lonza) in 0.5 TE× (10 mmol l^{-1} Tris:1 mmol L^{-1} EDTA, pH 8.0) and cast in plug molds. The agarose plugs were lysed for 2 hours, with shaking at 175 rpm at 54°C, in 5 mL of cell lysis solution (50 mmol L^{-1} Tris:50 mmol l^{-1} EDTA, pH 8.0 + 1% sarcosyl) (Lonza, Life Technologies, and Sigma-Aldrich, respectively) containing 25 µL of proteinase K stock solution (20 mg mL^{-1}). The plugs were then washed twice with sterile ultrapure water and 3 times with TE buffer for 10 minutes at 50 °C with shaking at 175 rpm. A small slice of the plug was digested with 50 U of enzyme (Promega) for 4 hours at 37°C. Fragments of DNA were separated in a 1% (w/v) SeaKem Gold agarose gel in 0.5× Tris-borate-EDTA buffer (Sigma-Aldrich) using a Chef-Mapper II system (Bio-Rad, Hercules, CA, USA) with the following conditions for both enzymes: low molecular weight (MW), 20 Kb; high MW, 215 Kb; run time, 19 hours. *Salmonella* Braenderup H9812 was used as the standard. Gels were stained with SYBR Safe DNA gel stain (Life Technologies) and photographed on a transilluminator. Image analysis was performed using Bionumerics v. 6.6 software (Applied Maths NV, Sint-Martens-Latem, Belgium). Pairwise comparisons and cluster analyses were conducted using the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm. The optimisation and position tolerances for the band analysis were set at 1%.

Results and discussion

A total of 14% (56/400) of porcine tonsils resulted positive for YE and all isolates belonged to bioserotype 4/O:3 (Table III).

All YE strains were susceptible to 6 of the 14 antimicrobial agents that were tested (amoxicillin/clavulanic acid, ciprofloxacin, gentamicin, trimethoprim/sulphamethoxazole) using broth microdilution.

Antimicrobial susceptibility tests revealed the well- known resistance of YE to ampicillin (100%), which is commonly described in literature (Bonke *et al.* 2011, Rusak *et al.* 2014), followed by 26.79%

Table III. *Results of multiplex PCRs of* Y. enterocolitica *strains in pigs (Central Italy).*

Y. enterocolitica bioserotype	Source	Number of strains	Chromosomal genes						Plasmid gene
			ystA	*irp2*	*16s*	*ail*	*inv*	*hemR*	*yadA*
4/O:3	Porcine tonsils	47	+	-	+	+	+	+	+
4/O:3	Porcine tonsils	1	+	-	+	+	+	-	-
4/O:3	Porcine tonsils	8	+	-	+	+	+	+	-

of strains resistant to streptomycin; 19.65% of strains resistant to sulfisoxazole; 16.08% of strains resistant to tetracycline; 14.30% of strains resistant to nalidixic acid; and 10.72% of strains resistant to chloramphenicol, some of which are usually used in yersiniosis treatment (Bonke *et al.* 2011, Fabrega and Vila 2011). Ampicillin resistance is probably due to the lack of expression of 2 *blaA* and *blaB* chromosomal genes encoding 2 β-lactamase. Previous studies indicate that YE strains expressing BlaA were susceptible to ampicillin, while strains not expressing BlaA were resistant to ampicillin. One explanation for this might be the different composition of the cell wall in different strains of *Yersinia* (Bonke *et al.* 2011, Sharma *et al.* 2004). In terms of multi-resistance profiles, 8 resistance patterns were observed in this study (Table IV). The most common resistance profile (AMP-CHL-NA-STR-SF) was observed in 8.93% of the isolates, followed by 7.14% of resistotype AMP-STR-SF-TET. Resistance to 3 antimicrobial was observed in 7.14% of the strains, while 3.57% shows AMP-STR profile. Only 2 isolates (3.57%) were resistant to 6 antimicrobials. Resistances to the antimicrobial agents that are tested have not changed recently due to the genetic stability of YE (Baumgartner *et al.* 2007, Bucher *et al.* 2008, Prats *et al.* 2000, Rastawicki *et al.* 2000).

The PCR profiles of the strains are shown in Table III. All strains that were biochemically identified previously were confirmed by PCR as YE (16S rRNA$^+$). Moreover, the *irp2*-negative PCR profiles confirmed the absence of YE biotype 1B. *Yersinia enterocolitica* bioserotype 4/O:3 isolates were divided into 3 groups: a total of 47 strains (83.92%) were positive for *ystA, ail, inv, hemR,* and *yadA* (*ystA*$^+$, *ail*$^+$, *inv*$^+$, *hemR*$^+$, *yadA*$^+$), 1 strain (1.79%) was positive for *ystA, ail*, and *inv* (*ystA*$^+$, *ail*$^+$, *inv*$^+$, *hemR*$^-$, *yadA*$^-$), and 8 isolates (14.29%) were positive for *ystA, ail, inv*, and *hemR* (*ystA*$^+$, *ail*$^+$, *inv*$^+$ *hemR*$^+$, *yadA*$^-$).

Several studies have described different multiplex PCR protocols for pathogenic YE (Aarts *et al.* 2001, Falcao *et al.* 2006, Harnett *et al.* 1996, Ibrahim *et al.* 1997, Jourdan *et al.* 2000, Kot *et al.* 2007, Thisted Lambertz and Danielsson-Tham 2005). However, some of these studies employed methods that did not analyse the 16S rRNA gene for species confirmations, others tested the *irp2* gene or *pYV* genes to identify the pathogenic biotype 1B, or limited their investigations to the *ail* gene (Wannet *et al.* 2001). Other studies described a simplex PCR for the detection of virulence genes (Gierczynski *et al.* 2009, Rasmussen *et al.* 1994, Thoerner *et al.* 2003). The advantage of the method used in our study is to rapidly identify both *Yersinia* species and the virulence genes, which is very useful for assessing the pathogenicity of the examined strains. Our results demonstrate that a multiplex PCR approach could simultaneously discriminate between highly pathogenic, pathogenic, and non-pathogenic strains. The inclusion of the *irp2* gene allowed us to detect the highly pathogenic biotype 1B, whereas the *ail* gene allowed us to identify non-pathogenic YE strains. The virulence of YE strains mostly depends on the presence of the pYV plasmid. Traditional chromosomal genes play a supporting role. In addition to the previously mentioned *irp2* and *ail* genes, our multiplex PCR can simultaneously amplify other important virulence genes, such as *ystA, inv, hemR*, and *yadA*, which are involved in the pathogenesis of this microorganism.

Table IV. *Resistance profiles of 56 isolates of* Y. enterocolitica *bioserotype 4/O:3.*

Resistance profiles	Total (%)
AMP	39/56 (69.64)
AMP-STR	2/56 (3.57)
AMP-STR-TET	2/56 (3.57)
AMP-CEF-SF	1/56 (1.79)
AMP-NA-TET	1/56 (1.79)
AMP-STR-SF-TET	4/56 (7.14)
AMP-CHL-NA-STR-SF	5/56 (8.93)
AMP-CHL-NA-STR-SF-TET	1/56 (1.79)
AMP-CEF-KAN-NA-STR-TET[a]	1/56 (1.79)

[a] AMP = ampicillin; STR = streptomycin; SF = sulfisoxazole; NA = nalidixic acid; TET = tetracycline; CEF = ceftriaxone; CHL = chloramphenicol; KAN = kanamycin.

The genomic profiles and dendrograms obtained by digesting the genomic DNA of theYE strains with XbaI and NotI are shown in Figure 1. We obtained 16 restriction endonuclease fingerprint profiles (pulsotypes), and their similarities ranged from 86.4 to 99.6%.

Pulsed-field gel electrophoresis identified 4 distinct clusters in all bioserotype 4:O:3 isolates from porcine tonsils, with a similarity of 95% among them. A single cluster was predominant, including 27 of the 56 pig isolates (48.21%), followed by a second, with 17 of the 56 pig isolates (30.35%). The other 2 clusters included, 6 (10.71%) and 4 (7.14%) isolates of porcine tonsils, respectively. Most of the isolates presented all of the virulence genes that were tested. Isolates without plasmid (*yadA*$^-$) were not included in the same clusters (Figure 1). The absence of pYV did not affect the analysis of the PFGE profiles, because digesting pYV with XbaI and NotI produces fragments of lower molecular weight than the minimum considered for the analysis (33.3 kb).

We used the PFGE technique to characterise and differentiate the YE bioserotype 4/O:3 isolates of pig tonsils.

In the past, PFGE has been reported to be useful for typing many bacteria, including YE (Buchrieser *et al.* 1994, Najdenski *et al.* 1994, Saken *et al.* 1994) and is

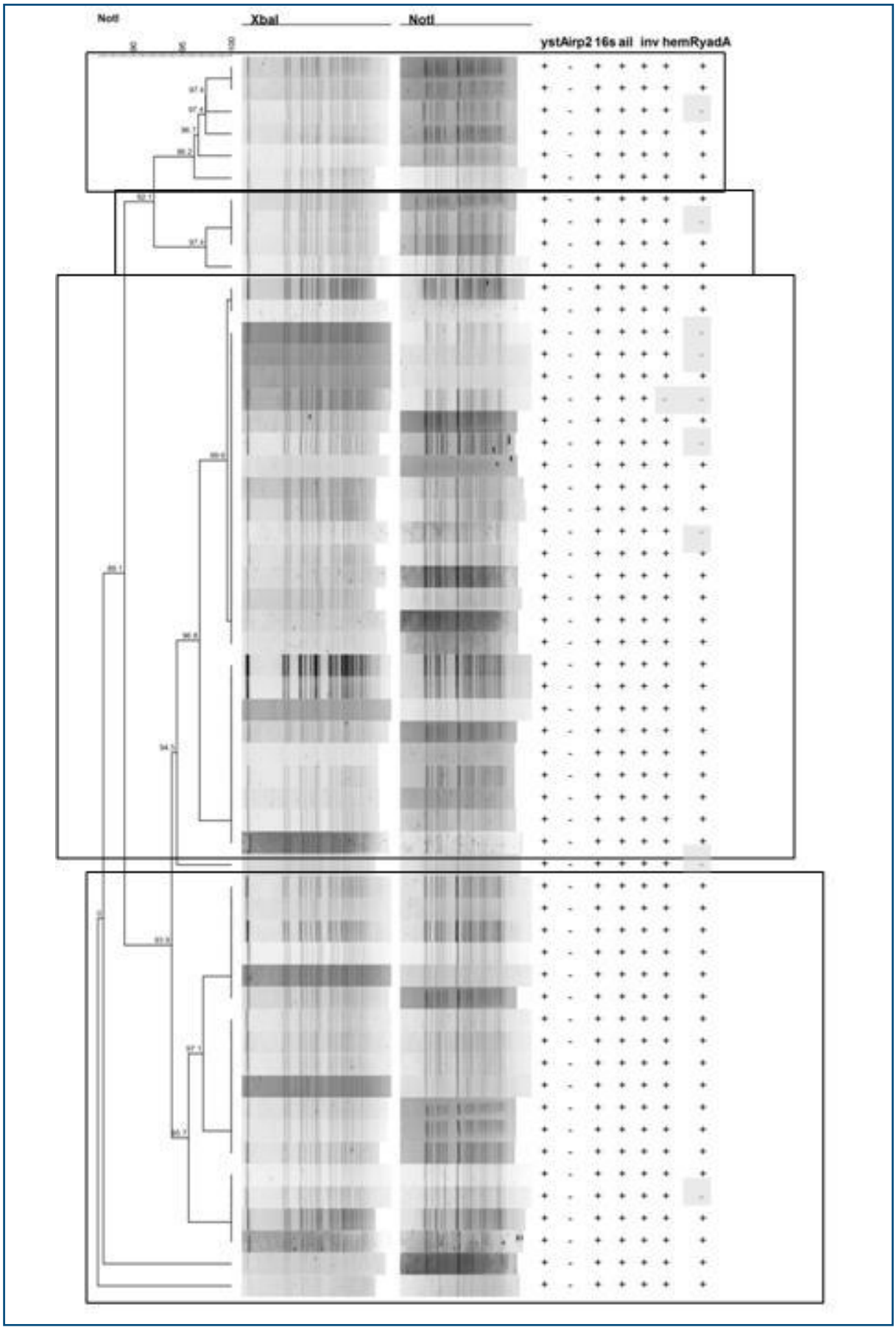

Figure 1. *Dendrogram obtained by digesting* Y. enterocolitica *bioserotype 4/O:3 genomic DNA with XbaI & NotI restriction enzymes.* Clusters with similarity to 95% are indicated by rectangles.

still valid in some situations (Thisted Lambertz and Danielsson-Tham 2005).

We found an high similarity (89.1% similarity) between the almost totality of the strains that differed in a very few bands. Our results showed a limited degree of genetic diversity among YE bioserotype 4/O:3 pig isolates, which is in agreement with many recent studies (Bhaduri *et al.* 2009, Gilpin *et al.* 2014, Paixao *et al.* 2013). This suggests that this method seems to have limited benefits for the genotyping of YE bioserotype 4/O:3 (Bari *et al.* 2011). We therefore recommend the use of alternative molecular methods for YE typing such as MLST, MLVA, and whole-genome sequencing in order to better understand the epidemiology of yersiniosis.

Human pathogenic YE bioserotype 4/O:3 strains have been frequently isolated from tonsils of healthy pigs, which are recognised to be an important portal of entry, as well as a site for multiplication and persistence for this bioserotype (EFSA 2015). Swine slaughter is an open process that offers many opportunities for the contamination of pig carcasses by zoonotic microorganisms such as YE. Tonsils can be a source of contamination for the head, tongue, and other offal, as well as for the carcass itself, when their removal is incomplete or tonsil-contaminated equipment is used for organ excision and carcass dressing. Prevention measures are therefore required in order to avoid the contamination of foodstuffs, which may lead to subsequent human infection. A number of biosecurity measures can be implemented at the primary stage of production in order to minimise the risk of contamination and infection.

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