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Yersinia enterocolitica of Porcine Origin: Carriage of Virulence Genes and Genotypic Diversity

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Abstract

Yersinia enterocolitica is an important foodborne pathogen, and pigs are recognized as a major reservoir and potential source of pathogenic strains to humans. A total of 172 *Y. enterocolitica* recovered from conventional and antimicrobial-free pig production systems from different geographic regions (North Carolina, Ohio, Michigan, Wisconsin, and Iowa) were investigated to determine their pathogenic significance to humans. Phenotypic and genotypic diversity of the isolates was assessed using antibiogram, serogrouping, and amplified fragment length polymorphism (AFLP). Carriage of chromosomal and plasmid-borne virulence genes were investigated using polymerase chain reaction. A total of 12 antimicrobial resistance patterns were identified. More than two-thirds (67.4%) of *Y. enterocolitica* were pan-susceptible, and 27.9% were resistant against β-lactams. The most predominant serogroup was O:3 (43%), followed by O:5 (25.6%) and O:9 (4.1%). Twenty-two of 172 (12.8%) isolates were found to carry *Yersinia* adhesion A (*yadA*), a virulence gene encoded on the *Yersinia* virulence plasmid. Sixty-nine (40.1%) isolates were found to carry *ail* gene. The *ystA* and *ystB* genes were detected in 77% and 26.2% of the strains, respectively. AFLP genotyping of isolates showed wide genotypic diversity and were grouped into nine clades with an overall genotypic similarity of 66.8–99.3%. AFLP analysis revealed that isolates from the same production system showed clonal relatedness, while more than one genotype of *Y. enterocolitica* circulates within a farm.

Introduction

Yersinia enterocolitica is an important foodborne pathogen known to cause gastrointestinal problems, with symptoms ranging from acute enteritis with fever to occasionally bloody watery diarrhea, particularly in children (Bottone, 1997). It is estimated to cause 116,716 cases of human illness annually in the United States, among which 90% may be attributed to foodborne transmission (Scallan *et al*., 2011).

Pigs are implicated as important reservoirs of *Yersinia enterocolitica* for humans (Fredriksson-Ahomaa *et al*., 2001; Bhaduri *et al*., 2009). Significant overlap in phenotypes and genotypes of human and pig strains have been reported (Fredriksson-Ahomaa *et al*., 2006, 2001; Falcao *et al*., 2006). However, all *Y. enterocolitica* strains are not pathogenic to immunocompetent individuals. Biogroups 1B, 2, 3, 4, and 5 are commonly associated with human infections, and some biogroup 1A have been isolated from humans with diarrhea and can cause opportunistic infections in immunocompromised individuals (Burnens *et al*., 1996; Bottone, 1997; Grant *et al*., 1998).

The pathogenicity of *Y. enterocolitica* depends primarily on the presence of virulence markers such as *Yersinia* adhesion A (*yadA*) and *Yersinia* outer membrane proteins (*yops*) located on a virulence plasmid (pYV- plasmid for *Yersinia* virulence), and virulence factors like attachment and invasion locus (*ail*), invasin (*inv*), thermostable enterotoxin (*ystA* and *ystB*) and mucoid *Yersinia* factor (*myfA*) encoded on a chromosome (Revell and Miller, 2001; Foultier and Cornelis, 2003). In this study, *yadA*, *ail*, *ystA,* and *ystB* were investigated based on their significance and role for clinical manifestations in humans (Ramamurthy *et al*., 1997; Revell and Miller, 2001).

Several DNA fingerprinting approaches including pulsed-field gel electrophoresis (PFGE), AFLP, and multilocus

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variable number tandem repeat analysis (MLVA) have previously been used to study genotypic diversity between human and animal strains and tracking outbreaks (Fredriksson-Ahomaa *et al*., 2001; Thisted Lambertz and Danielsson-Tham, 2005; Fearnley *et al*., 2005; Boghenbor *et al*., 2006; Falcao *et al*., 2006; Sihvonen *et al*., 2011). Here, we used AFLP to evaluate the genotypic diversity of *Y. enterocolitica* recovered from different swine production settings and geographic regions.

The objectives of the study include determining the phenotypic and genotypic diversity of *Y. enterocolitica* isolated from different pig production systems (conventional [Conv.] and antimicrobial-free [ABF] farms) and geographic regions (North Carolina, Ohio, Michigan, Iowa and Wisconsin) in the United States and characterizing the carriage of chromosomal and plasmid-encoded virulence genes known to cause human illness.

Materials and Methods

Bacterial strains

A total of 172 *Y. enterocolitica* isolates recovered from fecal samples and carcass swabs during the 3-year period 2002–2005 from various production types and geographic regions. Briefly, isolates were recovered from a multistate study designed to determine the prevalence of *Y. enterocolitica* pig on farm and at slaughter (carcass swabs at pre-evisceration, post-evisceration, and post-chill stages) representing two production types: ABF and Conv. in three regions (region 1, North Carolina: 10 ABF and 10 conv. Farms; region 2, Ohio and Michigan: 7 ABF and 9 conv. Farms; region 3, Wisconsin and Iowa: 13 ABF and 6 conv. farms). All *Y. enterocolitica* isolates recovered were included in this study.

We recovered 143 isolates (eight from region 1, 70 from region 2, and 65 from region 3) from an ABF production system where no antimicrobials were used post-weaning (used only therapeutically for sick pigs after removal to a separate barn), while 29 of the isolates (seven from region 1, one from region 2, and 21 from region 3) were isolated from conventional production system where antimicrobials are routinely used. Of 172 *Y. enterocolitica* isolates, 159 were recovered on-farm and 13 from the slaughter plant; collected at different stages of processing: pre-evisceration ($n=6$), post-evisceration ($n=2$), and post-chill ($n=5$). Isolation and identification of *Y. enterocolitica* were conducted as recommended by Funk *et al*. (1998). All presumptive isolates were further confirmed using polymerase chain reaction (PCR) as described by Wannet *et al*. (2001). Isolates were stored at −20°C until further phenotypic and genotypic characterizations.

Antimicrobial susceptibility

Antimicrobial susceptibility was determined using Sensititre semi-automated antimicrobial susceptibility system (Trek Diagnostics, West Lake, OH) following the Clinical Laboratory Standards Institute (CLSI) recommendations (NCCLS, 2002). The lists of antimicrobials and ranges of concentrations tested include the following: amikacin (0.5–32 μg/mL), amoxicillin/clavulanic acid (1/0.5–32/16 μg/mL), ampicillin (1–32 μg/mL), cefoxitin (0.5–32 μg/mL), ceftriaxone (0.5–64 μg/mL), chloramphenicol (2–32 μg/mL), ciprofloxacin (0.015–4 μg/mL), gentamicin (0.25–16 μg/mL), kanamycin (8–64 μg/mL), nalidixic acid (0.5–32 μg/mL), sulfisoxazole (16–512 μg/mL), tetracycline (4–32 μg/mL), and trimethoprim/sulfamethoxazole (0.12/2.38–4/76 μg/mL). Phenotyping was assessed based on the resistance pattern. Multidrug resistance (MDR) is defined here as resistance against two or more classes of antimicrobials.

Serogrouping

Serogrouping was performed by slide agglutination using O:3, O:5, O:8, and O:9 antisera (Denka Seiken, Tokyo, Japan) following the recommendation of the manufacturer.

PCR for virulence gene detection and aesculin test

All the isolates were tested for carriage of *ail*, *ystA*, *ystB*, and *yadA* virulence genes using PCR. Genomic DNA was purified using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's recommendation. Detection of *ail* and *yadA* genes was carried out using conventional PCR as previously described (Thoerner *et al*., 2003; Wannet *et al*., 2001). SYBR-Green–based real-time PCR was used for the detection of *ystA* and *ystB* genes, using primers adopted from Thoerner *et al*. (2003). The amplification was performed with the Stratagene Mx3005P QPCR apparatus (Stratagene Inc., La Jolla, CA). Briefly, one cycle of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing, and extension at 60°C.

Y. enterocolitica isolates were grown on bile aesculin agar (Becton Dickinson, Sparks, MD) and incubated at 30°C for 24 h to differentiate biotype 1A from the other biotypes. We used JB580v (8081) and NCTC 10460 as negative control strains for aesculin hydrolysis test.

Amplified fragment length polymorphism (AFLP) genotyping

We used a modification of the AFLP protocol described by Pandya *et al*. (2009). The amplified fragments were separated by capillary electrophoresis using CEQ 8000 genetic analyzer (Beckman Coulter, Fullerton, CA) as per the manufacturer recommendations. Fragments between 50 and 600 base pairs (bp) were scored using AFLP dominant scoring algorithm and analyzed using BioNumerics v4.6 software (Applied Maths, Kortrijk, Belgium). A genotypic similarity of 90% was used as a cut-off value for clustering. AFLP data generated from 13 isolates were removed from the analysis because of weak fluorescent signal.

Results

Phenotyping based on antimicrobial resistance pattern

Of 172 *Y. enterocolitica*, more than two-thirds (67.4%) were susceptible to all the tested antimicrobials. Forty-eight of the 172 (27.9 %) *Y. enterocolitica* isolates exhibited resistance to β-lactam antibiotics (either ampicillin or amoxicillin/clavulanic acid). A total of 12 antimicrobial resistance patterns were detected. Almost 20% (34 of 172) of the *Y. enterocolitica* isolates showed resistance to ampicillin only and 2.9% to sulfisoxazole only. Thirty-two of 143 isolates (22.4%) from ABF and two of the 29 (6.9%) from conventional production systems showed resistance to ampicillin only. None of the resistance patterns

Table 1. Serotype, Resistance Pattern, and Virulence Genes of *Yersinia enterocolitica* from Different Production Systems

		Virulence gene				*Prod. system* (n = *172*)	
Serogroup (n = *172*)	*R-pattern* (n = *172*)	ailA (n = *70*)	yadA (n = *22*)	ystA (n = *132*)	ystB (n = *37*)	*Conv.* (n = *29*)	*ABF* (n = *143*)
O:3 (74)	A (18)	+ (14)	+ (14)	+ (17)	+ (2)	1	17
	AT (3)	+ (3)	+ (3)	+ (3)	–	1	2
	AxACef (1)	–	–	+ (1)	–		1
	AxACT (1)	–	–	+ (1)	–	1	
	Sx (3)	+ (3)	+ (2)	+ (3)	+ (2)	1	2
	T (2)	–	–	+ (1)	–	1	1
	Pan-susceptible (46)	+ (26)	+ (3)	+ (37)	+ (2)	14	32
O:5 (44)	A (6)	+ (3)	–	+ (5)	+ (3)		6
	AxCefT (1)	+ (1)	–	–	+ (1)		1
	Sx (1)	–	–	+ (1)	+ (1)		1
	Pan-susceptible (36)	+ (9)	–	+ (26)	+ (5)	3	33
O:9 (7)	A (2)	–	–	+ (2)	–		2
	AGKT (1)	–	–	–	+ (1)		1
	AT (1)	+ (1)	–	+ (1)	+ (1)		1
	Sx (1)	–	–	+ (1)	–		1
	Pan-susceptible (2)	+ (1)	–	+ (1)	+ (2)	1	1
O:8 (5)	A (1)	–	–	+ (1)	–		1
	AxT (1)	–	–	+ (1)	–		1
	Pan-susceptible (3)	+ (2)	–	+ (3)	+ (1)		3
NT[a] (42)	A (7)	+ (2)	–	+ (5)	+ (1)	1	6
	AxA (2)	–	–	+ (1)	–		2
	AxACefT (1)	+ (1)	–	+ (1)	–		1
	AxAT (2)	–	–	–	+ (1)		2
	T (1)	–	–	–	+ (1)		1
	Pan-susceptible (29)	+ (4)	–	+ (20)	+ (13)	5	24

[a]NT, non-typable to antisera O:3, O:5, O:8, and O:9.
ABF, antimicrobial-free; Conv., conventional; A, ampicillin; Ax, amoxicillin/clavulanic acid; C, chloramphenicol; Cef, cefoxitin; G, gentamicin; K, kanamycin; Sx, sulfisoxazole; T, tetracycline.

were specific to one geographic region. The different resistance patterns are shown in Table 1.

Eleven (6.4%) *Y. enterocolitica* isolates were MDR. None of the isolates from the slaughter plant ($n = 13$) showed MDR. Seven of the 13 (53.8%) *Y. enterocolitica* recovered from the slaughter plant were pan-susceptible; three of the 13 (23.1%) showed resistance to ampicillin only, and the remaining three isolates showed resistance to sulfisoxazole only. None of the isolates were resistant to amikacin, ceftriaxone, ciprofloxacin, nalidixic acid, or trimethoprim/sulfamethoxazole.

Serogrouping

One hundred thirty of 172 (75.6%) *Y. enterocolitica* isolates were typable, conforming to one of the antisera tested: O:3, O:5, O:8, or O:9. The most common serogroup was O:3 (43%), followed by O:5 (25.6%) and O:9 (4.1%). Forty-two (24.4%) were untypable using O:3, O:5, O:8, and O:9 antisera. Thirteen of the 15 (86.7%) *Y. enterocolitica* recovered from region 1, 21 of 71 (29.6%) from region 2, and 40 of 86 (46.5%) from region 3 belonged to serogroup O:3. Two isolates from region 1, 22 from region 2, and 20 from region 3 were serogroup O:5. Isolates with serogroup O:8 and O:9 were identified only from region 2 and region 3. All, except serogroup O:8 (which were only recovered from ABF farms), were found in both ABF and conventional systems.

Carriage of virulence genes and aesculin test result

Twenty of 172 (11.6%) *Y. enterocolitica* tested were found to carry *yadA*. The *yadA* gene was detected only among serogroup O:3 (27% of the O:3 isolates): 18 of 143 (12.6%) of the isolates from ABF and two of the 29 (6.9%) of the isolates from conventional production systems. All *yadA*-positive *Y. enterocolitica* carried *ail* and *ystA* genes. Nineteen of 20 *yadA*-positive *Y. enterocolitica* isolates were aesculin negative. There was no significant difference in the carriage of *yadA* gene among *Y. enterocolitica* isolates recovered from different production systems ($p = 0.14$).

A total of 68 out of 172 (39.5%) were positive for the *ail* gene. Fifty-five of 68 *ail*-positive strains were aesculin negative, whereas 13 of 68 *ail*-positive strains were aesculin positive and thus could be of biotype 1A. Three of the four serogroups (other than O:8) were found to carry *ail* at different proportions (Table 2). Forty-six of 74 (62.2%) serogroup O:3, 13 of 44 (29.6%) serogroup O:5, and two of seven (28.6%) serogroup O:9 isolates carried *ail* gene. The *ystA* gene was the most common virulence gene detected in 132 (77%) of the isolates. This gene was detected in all serogroups: 63 of 74 (85%) serogroup O:3, 32 of 44 (73%) serogroup O:5, five of five (100%) serogroup O:8, and five of seven (71.4%) serogroup O:9. The *ystB* gene was detected in 45 of the 172 (26.2%) strains. Carriage of more than one virulence gene was detected in some isolates.

Table 2. Virulence Genes Commonly Associated with Pathogenic *Yersinia enterocolitica* Isolates

Serotype (n)	*Virulence gene* (n)		
	ail$^+$	ail$^+$ystA$^+$	ail$^+$ystA$^+$yadA$^+$
O:3 (74)	46	46	20
O:5 (44)	13	12	0
O:8 (5)	0	0	0
O:9 (7)	2	2	0
Un-typable (42)	7	5	0

Twenty of 172 (11.6%) *Y. enterocolitica* isolates carried *ail*, *ystA,* and *yadA* genes that are commonly associated with pathogenic strains. Sixty-five of the 68 (95.6%) *ail*-positive strains showed simultaneous carriage of the *ystA* gene (Table 2). There was significant association between *ail*- and *ystA*-positive *Y. enterocolitica* ($p < 0.002$).

Genotypic diversity using AFLP

A total of 159 *Y. enterocolitica* isolates (132 ABF and 27 conventional isolates) were genotyped using AFLP fingerprinting.

Genotyping of *Y. enterocolitica* strains showed wide diversity and were grouped into nine clades (A–I) at 77% genetic similarity threshold (Fig. 1). The overall genetic similarity of the 159 *Y. enterocolitica* isolates included in this study ranged between 66.8% and 99.3%. Isolates from different regions and production systems were represented in each of the clades. The majority of the isolates (127 of 159) were under clade A. The percent similarity of isolates in clade A ranged between 77.1% and 99.3%. With a 90% genetic similarity, a total of 15 clusters ($A_{1–15}$) were identified under clade A, whereas a total of 32 (20%) isolates were represented under clades B–I.

Under clade A, we found clustering of isolates from the same geographic region and production system in majority (80%, 12 of 15 clusters) of the cases. Three clusters (A_3, A_5, and A_6) represented isolates from both production systems. More than two-thirds of the isolates grouped together in each of the clusters were recovered from the same farm ($A_{1–4}$, A_8, $A_{13–15}$). It is also worth noting that there were isolates recovered from the same farm grouped in different clusters (A_2 and A_4, A_{14} and A_{15}), indicating the occurrence of more than one clone circulating in these farms. For example, cluster A_2 and A_4 primarily represent isolates recovered from region 2, farm 10; however, the clusters were distant from each other and only shared 83.7% similarity.

In clades B–I, isolates showed more genotypic heterogeneity. There were only a few clusters in each clade that have more than three isolates. The majority (18 of 32) of the isolates that fall under clades B–I were un-typable using antisera O:3, O:5, O:8, and O:9. None of the *Y. enterocolitica* strains recovered from fecal samples from farm and corresponding slaughterhouse carcass swab samples were genotypically clustered together.

Discussion

The major aims of this study were to investigate phenotypic and genotypic diversity, and carriage of virulence determinants among *Y. enterocolitica* recovered from pigs reared in different production systems and geographic regions. A total of 172 *Y. enterocolitica* isolates were included in the current study. Antimicrobial susceptibility testing against a panel of 13 antimicrobials revealed 67.4% of the isolates were pan-susceptible. More than a quarter of the strains showed resistance against β-lactams (either ampicillin or ampicillin-clavulanic acid combination). Multidrug resistance was uncommon and there was no significant difference in carriage of MDR isolates between ABF and conventional swine production system. Two of 29 (6.9%) of strains from conventional and 12 of 143 (8.4%) of strains from ABF isolates were MDR.

Yersinia pathogenesis depends on the presence of virulence genes located on the chromosome and plasmid (Revell and Miller, 2001). Twenty (11.6%) of our isolates were positive for *yadA, ail,* and *ystA* genes. The loss of pYV plasmid in *Y. enterocolitica* is a common phenomenon in delayed enrichment-based isolation and culture methods (Portnoy and Martinez, 1985). A delayed enrichment protocol was used in this study, and perhaps more isolates might originally be positive for *yadA* gene than detected if we had followed different isolation protocol. However, it is not possible to be certain when the plasmid is lost or whether the isolates had the pYV plasmid to begin with. Nineteen of 20 (95%) *yadA*-positive *Y. enterocolitica* isolates in the current study were aesculin negative and belonged to serogroup O:3. The common occurrence of serogroup O:3 in our study underscores the significance of pigs as important reservoirs of *Yersinia* of public health significance. Different studies indicated that *Y. enterocolitica* biogroup 4/O:3 is one of the most common serotypes from human yersiniosis cases (Bottone, 1999; Fredriksson-Ahomaa *et al*., 2001). Previous work done in the United States and elsewhere indicated that serotype O:3 is commonly recovered in pigs and pig products (Bhaduri and Wesley, 2006; Korte *et al*., 2004).

In the present study, 39.5% (68 out of 172) of the strains were found to carry the *ail* gene. More than 80% (55/68) of *ail*-positive strains were aesculin negative, indicating the potential pathogenic importance for humans. It is important to note that there are reports that showed the presence of the *ail* gene among *Y. enterocolitica* that are considered to be nonpathogenic like biotype 1A (Sihvonen *et al*., 2011; Kraushaar *et al*., 2011). In our study, 13 of 68 (19.1%) *ail*-positive strains were aesculin positive and thus considered to be nonpathogenic. Previous studies conducted in the United States and elsewhere have shown the presence of *ail*-positive *Y. enterocolitica* of pig origin (Gürtler *et al*., 2005; Bhaduri and Wesley, 2006; Bowman *et al*., 2007; Fredriksson-Ahomaa *et al*., 2007).

The *ystA* gene was the most common virulence gene detected in our study, where 77% of the isolates were positive. A previous study on the prevalence of the *yst* subtype among 304 *Y. enterocolitica* strains, from different sources and geographic regions, revealed that *ystA* was the most common heat-stable enterotoxin with 70.7% (Ramamurthy *et al*., 1997). The *yst* mediates the diarrhea observed in infants infected with *Y. enterocolitica* (Revell and Miller, 2001). This fact reiterates the potential pathogenic importance of *Y. enterocolitica* strains of porcine origin to human, from both conventional and ABF production systems. The *ystB* gene was detected in 45 of the 172 (26.2%) strains investigated in our study. Reports have shown the presence of the *ystB* gene in some non-pathogenic *Y. enterocolitica* and suggest it might be responsible for causing sporadic diarrhea (Pai *et al*., 1978; Ramamurthy *et al*., 1997).

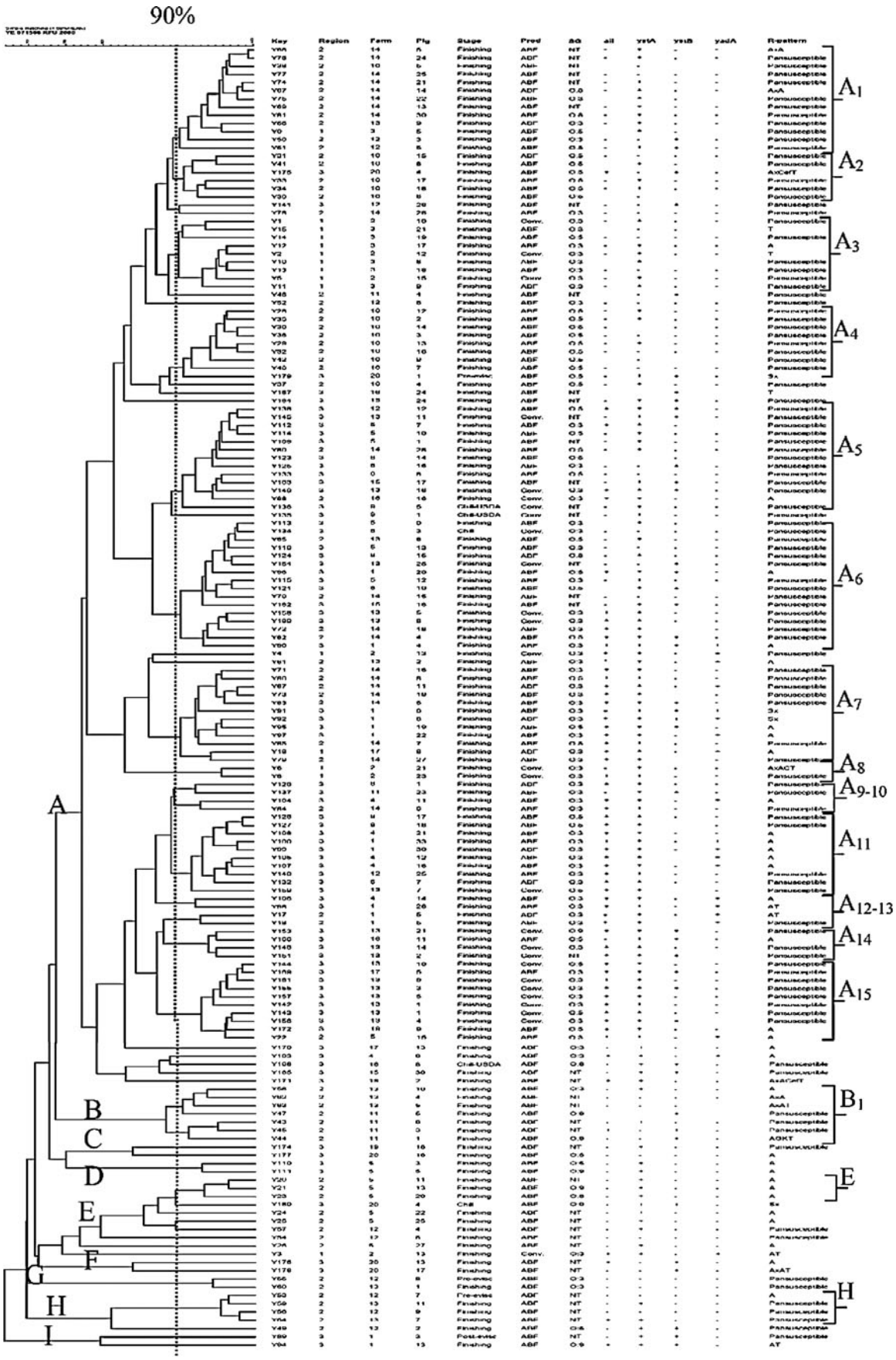

FIG. 1. Amplified fragment length polymorphism (AFLP) dendrogram of 159 *Yersinia enterocolitica* of pig origin. Isolates are clustered based on 90% simple matching coefficient genetic similarity level. SG, serogroup; NT, non-typable to antisera O:3, O:5, O:8, and O:9. Chill: carcass swab samples taken along the midline from ham to jowl at post-chill stages. Chill-USDA: carcass swab samples taken from three sites (ham, jowl, and belly) at post-chill stages.

Several DNA fingerprinting methods, including AFLP, have been used in subtyping of *Y. enterocolitica* to identify reservoirs of infection and for evaluating the similarity between human and animal serotypes (Fredriksson-Ahomaa *et al*., 2001; Thisted Lambertz and Danielsson-Tham, 2005; Fearnley *et al*., 2005; Boghenbor *et al*., 2006; Falcao *et al*., 2006; Bhaduri *et al*., 2009; Sihvonen *et al*., 2011). AFLP has high reproducibility and discriminatory power, and can be advantageous for large-scale epidemiologic investigations (Ross and Heuzenroeder, 2005; Gebreyes *et al*., 2006). The overall genotypic similarity of the 159 *Y. enterocolitica* isolates included in this study ranged between 66.8% and 99.3%. A total of nine clades and more than 15 clusters were observed. In some of the clusters, isolates from ABF and conventional production systems, and different geographic regions were grouped together, indicating high clonality of *Y. enterocolitica* strains regardless of production systems or geographic origins. This is in agreement with Bhaduri *et al.* (2009), which recently showed high clonality *Y. enterocolitica* of pig origin regardless of geographic region. PFGE characterization of *Y. enterocolitica* 4/O:3 recovered from tonsils of Bavarian slaughter pigs revealed the presence of one genotype, which is common to most of the farms (Fredriksson-Ahomaa *et al*., 2010). In this study, genotyping using AFLP showed that, in some instances, even isolates that originated from the same farm with the same phenotypes can show genetic diversity. This may be an indication that more than one clone circulated in these farms. Similarly, Laukkanen *et al*. (2009) demonstrated the presence of more than one genotype of *Y. enterocolitica* from a single farm or even from a single pig. A recent report by Fredriksson-Ahomaa *et al*. (2010) identified 31 genotypes of *Y. enterocolitica* 4/O:3 that are distributed and persisted for years in different swine farms. Although contamination of carcasses may occur during evisceration and chilling stages in the slaughter plant, none of the *Y. enterocolitica* strains recovered from farm and corresponding carcass swab samples, taken from the slaughter plant, were clustered together. This may indicate that there could be other sources of contamination of carcasses with *Y. enterocolitica* in the slaughter plant or that we could not pick them up during culturing of fecal samples at the farm. Laukkanen *et al*. (2009) investigated the transmission of pathogenic *Y. enterocolitica* from pigs to carcasses and pluck sets, and showed the presence of similar *Y. enterocolitica* 4/O:3 genotypes in animals on the farm and at the slaughterhouse and in carcasses.

In summary, *Y. enterocolitica* isolated from pigs carried virulence genes, regardless of production system or geographic region, and therefore, reduction of human *Yersinia* infection should include minimizing the carriage of pathogenic strains in pig herds. The high genetic diversity of *Y. enterocolitica* recovered from pig farms necessitates further investigation in identifying the source of contamination, both at the farm and slaughter plant.

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Disclosure Statement

No competing financial interests exist.

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