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Yersinia pseudotuberculosis with Limited Genetic Diversity Is a Common Finding in Tonsils of Fattening Pigs

TAINA NISKANEN,* MARIA FREDRIKSSON-AHOMAA, AND HANNU KORKEALA

Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, P.O. Box 57, FIN-00014, University of Helsinki, Helsinki, Finland

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ABSTRACT

A total of 425 pig tonsils, including 210 tonsils from fattening pigs and 215 from sows, from seven different abattoirs in Finland were studied for the occurrence of *Yersinia pseudotuberculosis* from 1999 to 2000. The mean prevalence of *Y. pseudotuberculosis* in fattening pig tonsils was 4%, varying from 0 to 10% between slaughterhouses. *Y. pseudotuberculosis* was not recovered from sow tonsils. All 30 *Y. pseudotuberculosis* isolates from eight pig tonsils were recovered after cold enrichment. Seventeen isolates from seven tonsils were found after cold enrichment for 14 days, followed by alkali treatment. *Y. pseudotuberculosis* was not isolated after direct plating, overnight enrichment, or selective enrichment. All 30 isolates belonged to bioserotype 2/O:3 and carried the *virF* gene in the virulence plasmid. The isolates exhibited calcium dependence and Congo red absorption. The pyrazinamidase test gave variable results. All isolates were characterized with pulsed-field gel electrophoresis (PFGE). Using *Spe*I, *Not*I, and *Xba*I enzymes, seven, five, and two different PFGE patterns were obtained, respectively. A total of 11 genotypes, gI to gXI, identified by a combination of the various *Spe*I, *Not*I, and *Xba*I profiles, were detected. Three pigs were found to carry more than one genotype. Overall, variations between PFGE patterns were small, indicating genetic homogeneity among pig strains of bioserotype 2/O:3.

Yersinia pseudotuberculosis is an uncommon cause of human yersiniosis *(19)*. It is primarily an animal pathogen, infecting humans only rarely *(24)*. In Finland, an annual rate of 10 cases per 1 million persons was reported in 1999 *(2)*. Infections due to *Y. pseudotuberculosis* may be acquired by ingestion of contaminated food or water, even though this bacterium has seldom been isolated from foods *(4, 10, 12, 35)*. Most *Y. pseudotuberculosis* infections are sporadic, and outbreaks have been rare, except in Japan and Finland *(2, 13, 29, 30, 35)*. Five outbreaks of *Y. pseudotuberculosis* have occurred between 1990 and 1999 in Finland *(2)*. In all of these cases, serotype O:3 was isolated from human feces *(2)*.

The epidemiology of *Y. pseudotuberculosis* is still poorly understood. *Y. pseudotuberculosis* has been isolated from domestic and wild animals with and without clinical symptoms *(8, 31, 37)*. *Y. pseudotuberculosis* has sporadically been isolated from tonsillar and fecal samples of clinically healthy pigs *(11, 20, 21, 27, 32, 38)*. However, no information is available on the prevalence of *Y. pseudotuberculosis* in pigs in Europe. As far as we know, the association between the age of pigs at slaughter and the prevalence of *Y. pseudotuberculosis* has not been studied. Furthermore, a need exists for efficient isolation and subtyping of *Y. pseudotuberculosis* strains for epidemiological studies.

Cold enrichment has been the most frequently used method to isolate *Y. pseudotuberculosis* from different samples *(3, 4, 16, 25, 36)*. Pathogenicity of the isolates has been confirmed in some studies using phenotypic tests, such as autoagglutination, calcium dependence, and Congo red uptake, all of which are characteristics associated with the virulence plasmid *(5, 17, 26, 33)*. Two polymerase chain reaction (PCR) methods have been developed to verify the pathogenicity of *Y. pseudotuberculosis* isolates *(15, 18, 39)*. The *virF* gene in the virulence plasmid and the *inv* gene in the chromosome have been used as target DNA in these methods.

Serotyping is the most commonly used method for the characterization of *Y. pseudotuberculosis*. However, to get more information on epidemiology, a need exists for the subtyping of isolates belonging to one serotype. Restriction enzyme analysis of plasmid and pulsed-field gel electrophoresis (PFGE) are the genotyping methods that have been previously used for the characterization of *Y. pseudotuberculosis* isolates *(9, 14, 17)*. PFGE was shown to be efficient in subtyping strains of the same serotype. However, little is known about the genetic variation of *Y. pseudotuberculosis* strains from different sources.

The purpose of this work was to study the prevalence of *Y. pseudotuberculosis* in fattening pigs and sows. Different isolation methods were used to determine the most efficient and productive alternative of recovering *Y. pseudotuberculosis* isolates. The genetic diversity of the isolates was studied by PFGE.

MATERIALS AND METHODS

Samples and sample preparation. Altogether, 425 pig tonsils were collected from seven different abattoirs in various parts of Finland from June 1999 to March 2000. Of these samples, 210

* Author for correspondence. Tel: 358-9-191 49701; Fax: 358-9-191 49718; E-mail: taina.niskanen@helsinki.fi.

were taken from fattening pigs and 215 from sows. The tonsils were cut out immediately after evisceration. One 10-g sample of tonsil tissue was homogenized in 90 ml Trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.), and another 10-g sample was homogenized in 90 ml phosphate-buffered saline (PMB, supplemented with 1% mannitol and 0.15% bile salts) for 1 min in a stomacher blender.

Isolation. *Y. pseudotuberculosis* was isolated by (i) direct plating on cefsulodin-irgasan-novobiocin agar (Yersinia Selective Agar [CIN] Base, Oxoid, Basingstoke, UK), (ii) overnight enrichment in TSB (Difco), (iii) selective enrichment in modified Rappaport broth (Merck, Darmstadt, Germany), and (iv) cold enrichment in PMB. In overnight enrichment, TSB homogenate was incubated at 22°C for 16 to 18 h. Then, 100 μl of this enrichment was inoculated into modified Rappaport broth and incubated for 3 days at 25°C. In cold enrichment, the PMB homogenates were incubated at 4°C for 7, 14, and 21 days. Alkali treatment (0.5 ml of the sample was mixed with 4.5 ml of 0.25% KOH solution for 20 s before being streaked onto CIN agar) was used after overnight enrichment and after 14 days of cold enrichment. CIN agar was used to isolate *Y. pseudotuberculosis* after every enrichment step. All inoculated CIN agar plates were incubated at 30°C for 18 to 20 h and then further at 22°C for 24 h. One to five suspect small, dark red colonies with a typical "bull's eye" appearance on the CIN agar were streaked onto blood agar plates for pure culture.

Identification, biotyping, and serotyping. One colony from the blood agar was inoculated onto a urea agar slant (Difco) and incubated for 1 day at 30°C. Isolates showing urea hydrolysis were further identified with API 20E (BioMérieux, Marcy l'Etoile, France) and incubated at 25°C for 18 to 20 h. All sucrose-negative isolates were further tested for xylose, trehalose, and salicin fermentation; esculin hydrolysis; and pyrazinamidase activity *(6)*. Biotyping of *Y. pseudotuberculosis* was based on reactions to citrate, melibiose, and raffinose *(33)*. The isolates were serotyped with slide agglutination using commercial O:1, O:2, O:3, O:4, O:5, and O:6 antisera (Denka Seiken, Tokyo, Japan).

Virulence-associated tests. To determine the pathogenicity, all sucrose-negative isolates were tested for calcium dependence and Congo red absorption with Congo red-magnesium oxalate agar (CR-MOX) *(23)* and the PCR assay *(18)*. The isolates were plated onto CR-MOX agar plates and incubated at 37°C for 24 h. Isolates were CR-MOX negative when only large colorless colonies were present; positive isolates produced small red colonies. The PCR assay targeting the *virF* gene on the virulence plasmid was used according to Nakajima et al. *(18)* with some modifications. In brief, five colonies from blood agar were suspended into 100 μl water. DNA was released by boiling the suspension for 10 min and then centrifuging at full speed (16,000 × *g*) for 3 min. Two microliters of the supernatant was used as a template in the PCR. The PCR reaction mixture volume was 50 μl and contained 1 U Dynazyme DNA polymerase (Finnzymes, Espoo, Finland), 200 μM of each dNTP (Finnzymes), and 0.3 μM of each primer (Pharmacia Biotech, Vantaa, Finland). The sequences of the oligonucleotide primers, based on the sequences of the *virF* gene, were 5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′. PCR was performed in a 16-well PTC-150 thermal cycler (MJ Research, Watertown, Mass.). The size of the amplified PCR product (about 600 bp) was determined in 0.8% agarose gel by comparison with DNA molecular weight marker VI (Boehringer Mannheim, Mannheim, Germany).

TABLE 1. *Prevalence of* Yersinia pseudotuberculosis *in tonsils of fattening pigs and sows in Finland*

	Tonsils of fattening pigs		Tonsils of sows	
Slaughter-house	No. of samples	No. of positive samples (%)	No. of samples	No. of positive samples (%)
A	30	1 (3)	30	0 (0)
B	30	1 (3)	29	0 (0)
C	30	3 (10)	35	0 (0)
D	30	0 (0)	30	0 (0)
E	30	1 (3)	30	0 (0)
F	30	0 (0)	30	0 (0)
G	30	2 (7)	31	0 (0)
Total	210	8 (4)	215	0 (0)

Characterization by PFGE. A total of 30 isolates were characterized by PFGE. DNA preparation and isolation were done according to Fredriksson-Ahomaa et al. *(11)*. Briefly, a single colony grown on blood agar was inoculated into 5 ml TSB and incubated overnight at room temperature. The cells in late log phase (18 h) were harvested from 2 ml TSB. The cells were washed once in 5 ml cold PIV (10 mM Tris [pH 7.5], 1 M NaCl) and then resuspended in 750 μl cold PIV. Of this cell suspension, 0.5 ml was mixed with an equal amount of 2% (wt/vol) low-melting-temperature agarose (InCert agarose, FMC Bioproducts, Rockland, Maine) and cast in GelSyringe dispensers (New England Biolabs, Beverly, Mass.). The plugs were lysed for 3 h in 2.5 ml lysis solution (6 mM Tris [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 20 μg/ml RNase, 1 mg/ml lysozyme) at 37°C with gentle shaking. The DNA isolation was completed with a single 2-h ESP (0.5 M EDTA [pH 8.0], 10% sodium lauryl sarcosine, 100 μg/ml proteinase K) wash at 50°C. The plugs were placed in fresh ESP solution and stored at 4°C. Before digestion, the plugs were washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA), the proteinase K was inactivated with Pefablock SC (Roche, Mannheim, Germany), and the plugs were further washed two times with TE buffer.

Restriction endonuclease digestion was performed according to manufacturers' instructions. The DNA was digested with three enzymes, *Not*I, *Xba*I, and *Spe*I (New England Biolabs). The samples were electrophoresed through a 1% (wt/vol) agarose gel (SeaKem Gold, FMC Bioproducts) in a 0.5× Tris-borate-EDTA buffer (Amresco, Solon, Ohio) at 12°C and 200 V using a Gene Navigator system (Pharmacia, Uppsala, Sweden) with a hexagonal electrode. Interpolation protocols ramping from 1 to 18 s for 20 h for *Not*I and from 1 to 15 s for 18 h for *Xba*I and *Spe*I were used. A low-range PFG marker (New England Biolabs) was used for fragment size determination. The gels were stained for 30 min in 1 liter running buffer containing 50 μl ethidium bromide (10 mg/ml) and were destained in running buffer until an appropriate contrast was obtained for digital imaging with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, Calif.). The banding patterns were interpreted visually. Isolates were considered to be different when a one-band difference was observed.

RESULTS

The prevalence of *Y. pseudotuberculosis* in the tonsils of fattening pigs was 4%, whereas all sow tonsils tested negative for the bacterium (Table 1). The prevalence of *Y.*

TABLE 2. *Characteristics of the sucrose-negative* Yersinia *isolates recovered from pig tonsils*

Tests	*Y. pseudotuberculosis* (30)[a]	*Y. kristensenii* (2)	*Y. enterocolitica* (11)
Biochemical identification			
Voges-Proskauer	−[b]	−	+
Rhamnose	−	−	−
Melibiose	−	−	−
Sorbitol	−	+	+
Indole	−	+	−
Xylose	+	+	−
Trehalose	v	+	+
NO_3	−	+	v
Pyrazinamidase	v	+	−
Esculin	+	−	−
Salicin	v	−	−
Pathogenicity			
CR-MOX agar	+	NS[c]	NS
PCR targeting *virF* gene	+	NS	NS

[a] Number of isolates.
[b] +, positive; −, negative; v, variable.
[c] NS, not studied.

pseudotuberculosis in fattening pig tonsils varied from 0 to 10% between slaughterhouses.

A total of 43 urea-positive, sucrose-negative isolates of typical "bull's eye" appearance on the CIN agar plates were recovered. Using biochemical tests, 30, 2, and 11 isolates were identified as *Y. pseudotuberculosis, Yersinia kristensenii,* and *Yersinia enterocolitica,* respectively (Table 2). The other *Yersinia* spp. grew faster on CIN agar plates than *Y. pseudotuberculosis.* Some of the CIN agar plates were overgrown with atypical colonies of "bull's eye" appearance. All isolated *Y. pseudotuberculosis* strains were esculin positive, and salicin and pyrazinamidase reactions varied. All isolates showed calcium dependence and Congo red absorption on CR-MOX agar plates (Table 2). The *virF* gene was detected in all isolates, with a strongly predominant band at about 600 kb in PCR (Fig. 1). All *Y. pseudotuberculosis* isolates belonged to biotype 2, with negative melibiose, citrate, and raffinose tests agglutinated with O:3 antiserum.

Cold enrichment followed by alkali treatment was the most productive isolation method. From seven positive samples, 17 isolates were recovered after treatment with this method, with only one *Y. pseudotuberculosis*-positive sample not recovered (Table 3). A total of three and four positive samples were found after cold enrichment for 7 and 21 days, respectively. *Y. pseudotuberculosis* was not isolated after direct plating, overnight enrichment, or selective enrichment.

Altogether, seven, five, and two different PFGE patterns were obtained with *Spe*I, *Not*I, and *Xba*I enzymes, respectively, when 30 isolates of *Y. pseudotuberculosis* were characterized (Table 4). The variations between the PFGE patterns were small, especially when the *Xba*I enzyme was used. One- to six-band differences were observed with *Spe*I (Fig. 2), but overall, the variations were small. A total of 11 genotypes, identified by combining the various *Spe*I, *Not*I, and *Xba*I digestion profiles, were obtained. The most common genotypes were gI, found from three of five slaughterhouses, and gIII, found from only one slaughterhouse but from three pigs, originating from different farms. For four of eight pigs, more than one genotype was isolated from the tonsils.

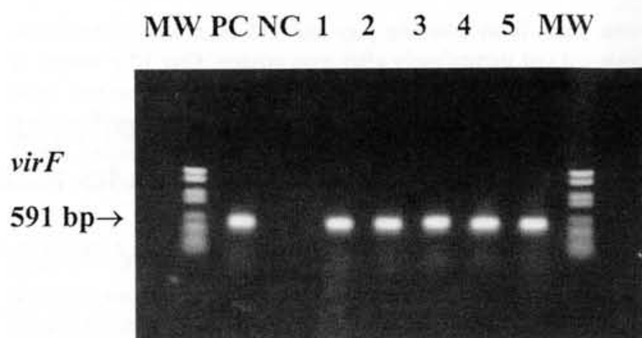

FIGURE 1. *Positive* virF *patterns using PCR. Lane 1 through 5 results obtained by examination of five* Yersinia pseudotuberculosis *isolates from pig tonsils. MW, molecular weight marker; PC, positive control; NC, negative control.*

DISCUSSION

In Finland, fattening pigs carry pathogenic *Y. pseudotuberculosis* of bioserotype 2/O:3 in the tonsils. The fairly low isolation rate of 4% is in accordance with earlier studies. In Germany, Weber and Knapp *(38)* isolated *Y. pseudotuberculosis* from 28 (6%) of 480 pig tonsils in one slaughterhouse. In Japan, Fukushima et al. *(11)* recovered *Y. pseudotuberculosis* from 33 (3%) of 1,200 pigs by a swabbing of the oral cavity, and Shiozawa et al. *(27)* re-

TABLE 3. Yersinia pseudotuberculosis *isolates recovered by different isolation methods*

Pig	No. of isolates recovered					
	Direct plating[a]	Overnight enrichment[b]	Selective enrichment[c]	Cold enrichment[d] 7 days	Cold enrichment[d] 14 days[e]	Cold enrichment[d] 21 days
A26	0	0	0	0	0	1
B3	0	0	0	0	5	0
C37	0	0	0	2	3	1
C40	0	0	0	0	2	0
C45	0	0	0	0	1	0
E23	0	0	0	0	1	0
G11	0	0	0	1	1	1
G21	0	0	0	3	4	4
Total	0	0	0	6	17	7

[a] Direct plating onto CIN agar plate.
[b] Overnight enrichment in TSB at 22°C for 16 to 18 h.
[c] Selective enrichment in modified Rappaport broth at 25°C for 3 days.
[d] Cold enrichment in PMB at 4°C for 7, 14, and 21 days.
[e] KOH treatment in 0.25% solution for 20 s before plating.

TABLE 4. *Different genotypes obtained with combinations of* SpeI, NotI, *and* XbaI *digestion profiles of 30* Yersinia pseudotuberculosis *bioserotype 2/O:3 isolates from pig tonsils*

Slaughter-house	Pig	No. of iso-lates	Enrichment step	PFGE pattern			Geno-type
				*Spe*I	*Not*I	*Xba*I	
A	A26	1	PMB21[a]	1	2	1	I
B	B3	5	PMB14[b]	2	4	2	II
C	C37	1	PMB7[c]	3	1	2	III
	C37	1	PMB7	3	4	2	IV
	C37	1	PMB14	3	1	2	III
	C37	2	PMB14	3	4	2	IV
	C37	1	PMB21	7	1	2	V
	C40	1	PMB14	3	1	2	III
	C40	1	PMB14	3	4	2	IV
	C45	1	PMB14	3	1	2	III
E	E23	1	PMB14	1	2	1	I
G	G11	1	PMB7	4	3	2	VI
	G11	1	PMB14	4	4	2	VII
	G11	1	PMB21	5	3	2	VIII
	G21	1	PMB7	6	2	1	IX
	G21	2	PMB7	1	4	1	X
	G21	1	PMB14	1	2	1	I
	G21	3	PMB14	1	4	1	X
	G21	1	PMB21	1	2	1	I
	G21	2	PMB21	1	4	1	X
	G21	1	PMB21	1	5	1	XI

[a] Cold enrichment in PMB at 4°C for 21 days.
[b] Cold enrichment in PMB at 4°C for 14 days followed by alkali treatment.
[c] Cold enrichment in PMB at 4°C for 7 days.

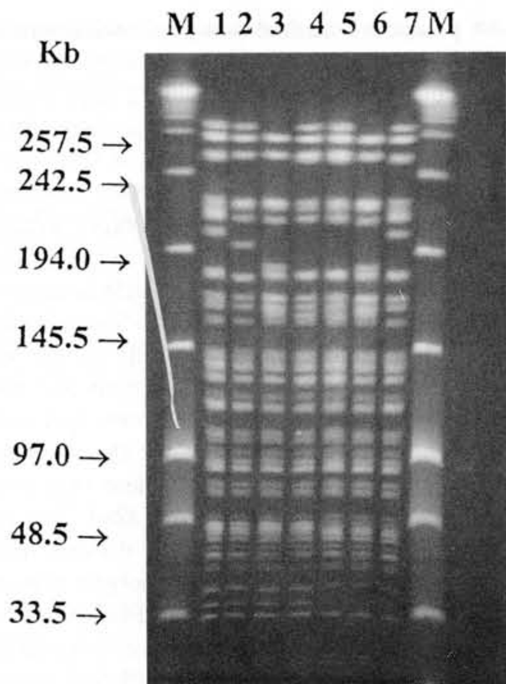

FIGURE 2. *Seven different* SpeI *profiles for 30 isolates of* Yersinia pseudotuberculosis *belonging to bioserotype 2/O:3 from pig tonsils; M, low range PFG marker 1.*

covered the bacterium from 3 (2%) of 150 pig tonsils. The isolation rates varied between slaughterhouses in Finland. In one slaughterhouse, an isolation rate of 10% was obtained, and in two of the seven slaughterhouses, no *Y. pseudotuberculosis* was found. This may be due to different prevalences of infected herds in different areas. *Y. pseudotuberculosis* was not isolated from sow tonsils. The reason for this may be that fattening pigs are more sensitive to *Y. pseudotuberculosis* infections than sows, which have developed natural resistance against the bacteria.

All *Y. pseudotuberculosis* isolates were shown to be pathogenic when one genotypic test and two phenotypic tests were used. Virulence plasmid-associated characteristics including calcium dependence and Congo red absorption correlated with the presence of the *virF* gene, which is located in the virulence plasmid. The virulence plasmid has been shown to be essential for full virulence of all pathogenic *Yersinia* spp. *(22)*. The PCR assay was a rapid and convenient method to confirm the pathogenicity of the isolates. It was also easier to interpret than calcium dependence and Congo red absorption on CR-MOX agar plates.

The bioserotype 2/O:3 was the only type recovered from pig tonsils in Finland. Serotype O:3 has been found in pig tonsils and feces samples, especially in Japan *(11, 27, 35)*. In other parts of Europe, other serotypes, such as O:1 and O:2, have been recovered more commonly *(21, 38)*. Biotyping of *Y. pseudotuberculosis* has been used in a limited number of studies *(1, 17, 33)*, and bioserotype 2/O:3 has been shown to be common in Brazil among cattle and buffaloes *(17)*. In contrast to our results, however, Tsubokura et al. *(34)* reported that all melibiose-negative isolates of serotype O:3 isolated from pigs were nonpathogenic.

Pyrazinamidase activity, esculin hydrolysis, and salicin fermentation have been used to differentiate pathogenic *Y. enterocolitica* isolates from nonpathogenic ones *(6)*, with reactions being negative in pathogenic *Y. enterocolitica* isolates. However, all *Y. pseudotuberculosis* isolates were positive for esculin reaction, and salicin and pyrazinamidase reactions varied, indicating that these tests are unsuitable for the identification of pathogenic *Y. pseudotuberculosis* isolates, in accordance with the study by Martins et al. *(17)*. Sucrose-negative *Y. enterocolitica* isolates recovered in our study could be differentiated from *Y. pseudotuberculosis* with sorbitol, xylose, and esculin tests (Table 2).

Several culture methods for the isolation of *Y. pseudotuberculosis* were compared. All isolates were recovered after cold enrichment, and no isolates were found with direct plating or after overnight and selective enrichment. The CIN agar plates were overgrown with bacteria with a "bull's eye" appearance, including other *Yersinia* spp., after overnight enrichment in TSB. This made it impossible to find slow-growing *Y. pseudotuberculosis* isolates with this method, even when alkali treatment was used (data not shown). Sierra et al. *(28)* have described the background flora from CIN agar and the different species suspected of harboring *Yersinia* during the examination of lamb. In our study, *Y. pseudotuberculosis* was not isolated after selective enrichment in modified Rappaport broth, widely used in the isolation of *Y. enterocolitica (24)*. This method was shown to be unsuitable for the isolation of *Y. pseudotuberculosis*.

The most productive method was cold enrichment for 14 days followed by alkali treatment. Possibly, the isolation rates after cold enrichment for 7 and 21 days would also have been higher if alkali treatment had been used after these enrichment steps as well.

Y. pseudotuberculosis isolates belonging to bioserotype 2/O:3 were subdivided into several genotypes with PFGE. The genotypes gI and gIII were detected in pigs originating from different farms; some genotypes might geographically be more widely distributed than others. Some of the pigs harbored two or three different strains in the tonsils. Sources of *Y. pseudotuberculosis* infections and possible reinfections in pigs are not yet well known. *Spe*I and *Not*I were shown to be suitable enzymes for characterizing the *Y. pseudotuberculosis* isolates. With these two enzymes, more genotypes were found than with *Xba*I. The isolates cleaved with the *Xba*I enzyme were divided into two groups, but the number of different genotypes was not increased. The differences between the PFGE patterns were clear. Although Iteman et al. *(14)* have characterized *Y. pseudotuberculosis* isolates efficiently with *Not*I enzyme, in our study, *Spe*I had better discriminatory power. However, pattern variations were small, indicating limited genetic diversity among pig strains belonging to bioserotype 2/O:3.

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