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Molecular typing of *Yersinia pseudotuberculosis* strains isolated from livestock in Brazil

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ABSTRACT. *Yersinia pseudotuberculosis* can infect a broad range of animals. In Brazil, this bacterium has been isolated from healthy and sick animals from sporadic cases and outbreaks of hemorrhagic gastroenteritis among livestock. However, the molecular diversity of these isolates is little understood. In this study, we used multilocus sequence typing, enterobacterial repetitive intergenic consensus PCR and pulsed-field gel electrophoresis to genotype 40 *Y. pseudotuberculosis* strains belonging to bio-serogroups 1/O:1a and 2/O:3 isolated between 1982 and 1990 in the southern region of Brazil. All three methodologies clustered the strains into two main clusters according to their bio-serogroups. Good correlations were observed between the clusters and the pathogenic potential of the strains. No correlation among the strains was observed according to geographical origin, host, place, or year of isolation. The grouping of the *Y. pseudotuberculosis* isolated in Brazil determined by these assays leads us to suggest that Brazilian livestock

harbor two subpopulations of *Y. pseudotuberculosis*.

Key words: *Yersina pseudotuberculosis*; Molecular typing; PFGE; Multilocus sequence typing; ERIC-PCR

INTRODUCTION

Yersinia pseudotuberculosis is one of the most important pathogenic species of the genus *Yersinia* and is able to infect a wide range of hosts, causing enteric disease in humans and animals in both sporadic and outbreak cases (Fukushima et al., 2001; Fredriksson-Ahomaa, 2007). The pathogenicity of *Y. pseudotuberculosis* is determined by the presence of the *Yersinia* virulence plasmid (pYV), the high-pathogenicity island (HPI), and the *Y. pseudotuberculosis*-derived mitogen (YPM) (Fukushima et al., 2001).

Y. pseudotuberculosis is widely spread in the environment, where it can survive for a long time. Soil and water may be contaminated by the feces of infected animals, mainly rodents and birds (Fredriksson-Ahomaa, 2007). The transmission of *Y. pseudotuberculosis* occurs primarily through the fecal-oral route. The clinical manifestations can vary from mild enteritis to extra-intestinal symptoms and septicemia (Fredriksson-Ahomaa, 2007; Schriefer and Petersen, 2011). The most common symptoms are fever, abdominal pain, vomiting, diarrhea, and bloody stools. Although septicemia rarely occurs, the mortality rate for sepsis can be as high as 75%, even with the use of antibiotics (Smego et al., 1999; Schriefer and Petersen, 2011).

Y. pseudotuberculosis strains can be divided into four biotypes according to their ability to ferment melibiose and raffinose and to utilize Simmons' citrate. In addition, it can be typed into 15 serogroups (O:1 to O:15) on the basis of antigenic variations in their cell-wall lipopolysaccharide. In turn, these serogroups can be subdivided into 10 subgroups (1a, 1b, 1c, 2a, 2b, 2c, 4a, 4b, 5a, and 5b) (Schriefer and Petersen, 2011). Alternatively, *Y. pseudotuberculosis* can also be grouped into six genetic groups (G1-G6) on the basis of the presence of pYV, HPI, and YPM (Fukushima et al., 2001).

In Brazil, *Y. pseudotuberculosis* strains have been isolated from healthy and sick animals from 1982 until 2009 in both sporadic and outbreak cases of hemorrhagic gastroenteritis among livestock. Healthy animals usually have low levels of *Y. pseudotuberculosis* in their intestinal contents (Estima and Riet-Corrêa, 1993) in contrast to the high levels observed in sick animals (Warth et al., 2012). This bacterium has been responsible for more than 100 reported episodes of hemorrhagic gastroenteritis in *Bos taurus indicus* and *Bubalus bubalis* and only one suspected case in *Sus scrofa domesticus*. However, the isolation of this pathogen from humans has never been reported in this country (Falcão et al., 2008; Warth et al., 2012).

Little is known about the molecular epidemiology of *Y. pseudotuberculosis* in Brazil. To our knowledge, only two studies concerning the molecular epidemiology of *Y. pseudotuberculosis* strains isolated in this country have been published (Martins et al., 2007; Souza and Falcão, 2012). In the study of Martins et al. (2007), ribotyping was performed but was not able to establish any epidemiological correlation between the strains. In the study of Souza and Falcão (2012), the high-resolution melting analysis technique grouped the strains according to their bio-serogroups.

To obtain further epidemiological information about the *Y. pseudotuberculosis* isolated in Brazil, we conducted this study using multilocus sequence typing (MLST), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), and pulsed-field gel electrophoresis (PFGE) assays to assess the genotypic diversity of strains of this pathogen isolated in this country.

MATERIAL AND METHODS

Bacterial strains

A total of 40 *Y. pseudotuberculosis* strains selected from the collection of "Laboratório Nacional de Referência em *Yersinia* spp. outras que *Y. pestis*" were studied. All of these strains were isolated from healthy and sick buffaloes, cattle, and pigs from both sporadic cases and outbreaks of hemorrhagic gastroenteritis in the southern region of Brazil and are presented in Table 1.

Table 1. Origin and types of the 40 *Yersinia pseudotuberculosis* strains used in the MLST, ERIC-PCR, and PFGE assays.

Strains	Source	Material	Biotype	Serogroup	Year	Place
P1	Sick buffalo	Organs	2	O:3	1982	Curitiba/PR
P2	Sick bovine	Intestinal loop	2	O:3	1983	Curitiba/PR
P4	Sick bovine	Diarrheic feces	2	O:3	1984	Curitiba/PR
P8	Sick bovine	Diarrheic feces	2	O:3	1984	Londrina/PR
P10	Sick bovine	Diarrheic feces	2	O:3	1984	Curitiba/PR
P14	Sick bovine	Diarrheic feces	2	O:3	1984	Curitiba/PR
P15	Sick bovine	Diarrheic feces	2	O:3	1984	Curitiba/PR
P21	Sick swine	Diarrheic feces	2	O:3	1985	Londrina/PR
P22	Sick bovine	Diarrheic feces	2	O:3	1985	Londrina/PR
P26	Sick bovine	Small intestine	2	O:3	1985	Curitiba/PR
P28	Sick bovine	Diarrheic feces	2	O:3	1987	Curitiba/PR
P30	Sick buffalo	Mesenteric ganglion	1	O:1a	1989	Pelotas/RS
P31	Sick buffalo	Mesenteric ganglion	1	O:1a	1989	Pelotas/RS
P32	Sick buffalo	Mesenteric ganglion	1	O:1a	1989	Pelotas/RS
P34	Sick bovine	Diarrheic feces	2	O:3	1989	Curitiba/PR
P35	Sick bovine	Small intestine	2	O:3	1989	Curitiba/PR
P37	Sick bovine	Diarrheic feces	2	O:3	1989	Curitiba/PR
P40	Sick bovine	Diarrheic feces	2	O:3	1989	Curitiba/PR
P43	Sick bovine	Diarrheic feces	2	O:3	1989	Curitiba/PR
P45	Sick bovine	Small intestine	2	O:3	1989	Curitiba/PR
P46	Sick bovine	Large intestine	2	O:3	1989	Curitiba/PR
P52	Sick bovine	Large intestine	2	O:3	1989	Curitiba/PR
P57	Sick bovine	Small intestine	2	O:3	1989	Curitiba/PR
P61	Sick bovine	Diarrheic feces	2	O:3	1989	Curitiba/PR
P67	Sick bovine	Diarrheic feces	2	O:3	1990	Curitiba/PR
P70	Sick bovine	Diarrheic feces	2	O:3	1990	Curitiba/PR
P71	Sick bovine	Small intestine	2	O:3	1990	Curitiba/PR
P75	Sick bovine	Diarrheic feces	2	O:3	1990	Curitiba/PR
P78	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P80	Sick buffalo	Diarrheic feces	2	O:3	1990	Pelotas/RS
P84	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P85	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P90	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P94	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P95	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P99	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P100	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P101	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P104	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS
P105	Healthy buffalo	Feces	2	O:3	1990	Pelotas/RS

DNA extraction

Each of the 40 *Y. pseudotuberculosis* strains listed in Table 1 was cultured in brain

heart infusion broth and incubated overnight at 28ºC. Genomic DNA was extracted from the growing cells as described by Falcão et al. (2006). The DNA concentration of each sample was determined using a NanoDrop 1000 (Thermo Scientific, San Jose, CA, USA), and the purity of the DNA was estimated as described by Sambrook and Russell (2001).

MLST

The MLST assay was performed using 7 housekeeping genes (*adk*, *argA*, *aroA*, *glnA*, *thrA*, *tmk*, and *trpE*) according to the method of Laukkanen-Ninios et al. (2011). A detailed protocol for the MLST procedure, including the primers used for the amplification of the seven housekeeping genes and the annealing temperature is available in the *Y. pseudotuberculosis* MLST database (http://mlst.ucc.ie/mlst/dbs/Ypseudotuberculosis). All amplifications were performed in a total volume of 50 µL as described by Souza et al. (2010). A sample of the complete mix without the DNA was used as a negative control in all runs. Automated DNA sequencing was performed with a MegaBACE 1000 DNA Sequencer (GE HealthCare, Little Chalfont, UK) using the primer set described in the *Y. pseudotuberculosis* MLST database. Each forward and reverse strand was sequenced at least three times. For each strain, consensus sequences were obtained using the ChromasPro version 1.5 software package (Technelysium Pty Ltd., Helensvale, Australia) for later analysis online using the *Y. pseudotuberculosis* MLST database. Each unique sequence was assigned as a different allele number using the MLST database, and the combination of the seven alleles, one for each target gene, provided the sequence type (ST). The eBURST V3 algorithm (http://eburst.mlst.net/) (Feil et al., 2004) was used to determine the genetic relationships between all STs related to strains available in the *Y. pseudotuberculosis* MLST database, including the strains used in this study.

ERIC-PCR

The ERIC-PCR assay was performed as described by Souza et al. (2010) using the primer pair described by Versalovic et al. (1991). The ERIC-PCR assay was repeated twice for each strain to verify the reproducibility of the results. The ERIC-PCR amplicons were resolved into bands by 1.5% agarose gel electrophoresis. The gels were stained with 0.5 µg/mL ethidium bromide and visualized under UV light. Only bands representing amplicons between 100 and 5000 bp in size were included in the analysis. A standard molecular weight ladder (1-kb Plus DNA Ladder - Invitrogen, Carlsbad CA, USA) was included three times on each gel to facilitate the normalization of the images and thus allows valid comparisons of fingerprints on different gels. Clustering was performed with the BioNumerics 7.0 software package (Applied Maths NV, Sint-Martens-Latem, Belgium) using the unweighted pair group method with arithmetic mean (UPGMA) method and the Dice similarity coefficient.

PFGE

The genomic DNAs of all strains were prepared in agarose plugs as described by Souza et al. (2010). The plugs were digested with 40 U *Xba*I (Invitrogen) for 18 h. Macrorestriction fragments were resolved by contour-clamped homogeneous electric field electrophoresis in a CHEF DR® III Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Berkeley, CA, USA) with an electric field of 6 V/cm and an angle of 120°. The migration of fragments was performed at 14°C in 0.5X TBE buffer (4.5 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) and 1.0% ultra-pure pulsed-field agarose (Bio-Rad Laboratories). The pulse times

ranged from 1 to 10 s for 29 h, as described by Guiyoule et al. (1997). A standard molecular weight ladder (Lambda Ladder PFG Marker, New England BioLabs, Ipswich, MA, USA) was included three times on each gel to facilitate the comparison of the fingerprints from different gels. Only bands representing fragments between 48.5 and 194.0 kb in size were included in the analysis. The relatedness between the PFGE profiles was determined with the BioNumerics 7.0 software package (Applied Maths NV). A similarity dendrogram was constructed using the UPGMA method with the Dice similarity coefficient.

RESULTS AND DISCUSSION

Despite the fact that several reports regarding the genotypic diversity of *Yersinia* species, including *Y. pseudotuberculosis* strains isolated around the world have been published, the molecular epidemiology of *Y. pseudotuberculosis* strains isolated from livestock in Brazil remains unclear (Kim et al., 2003; Voskressenskaya et al., 2005; Martins et al., 2007; Souza et al., 2010; Ch'ng et al., 2011; Laukkanen-Ninios et al., 2011).

To elucidate the epidemiological relationships between the *Y. pseudotuberculosis* strains isolated in Brazil, we decided to assess the molecular relatedness of these bacteria using the MLST, ERIC-PCR, and PFGE.

As shown in Figure 1, the eBURST analysis based on MLST data clustered the 40 *Y. pseudotuberculosis* strains tested into two STs. All 37 *Y. pseudotuberculosis* strains belonging to bio-serogroup 2/O:3 were grouped into ST 19, and the three *Y. pseudotuberculosis* strains belonging to bio-serogroup 1/O:1a were grouped into ST 42.

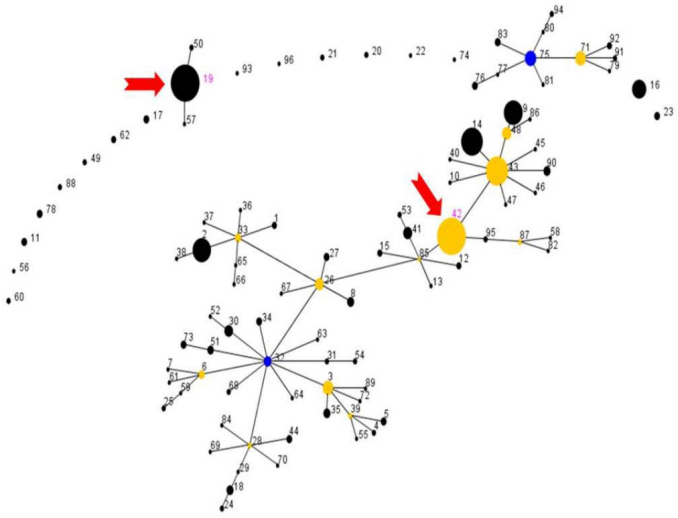

Figure 1. eBURST diagram showing the genetic relationships among the 40 *Yersinia pseudotuberculosis* strains studied and all the strains currently listed in the *Y. pseudotuberculosis* MLST database. Each sequence type (ST) is represented by a dot with a size proportional to the number of *Y. pseudotuberculosis* strains belonging to that ST, considering all strains in the database. The arrows indicate the STs that are related to the *Y. pseudotuberculosis* strains isolated in Brazil. The strains used in this study that belonged to the 1/O:1a bio-serogroup were classified as ST 42, and the strains that belonged to the 2/O:3 bio-serogroup were classified as ST 19.

In a global analysis of all *Y. pseudotuberculosis* strains listed in the *Y. pseudotuberculosis* MLST database including the strains of this study, it was observed that the *Y. pseudotuberculosis* O:3 strains were distributed into 17 different STs (3, 4, 14, 19, 25, 30, 32, 43, 50, 57, 58, 59, 60, 61, 62, 63, and 64). These strains were isolated globally from human and nonhuman sources and are described as having low pathogenic potential (Fukushima et al., 2001; Fredriksson-Ahomaa, 2007; Laukkanen-Ninios et al., 2011). Despite the low pathogenicity characteristic of *Y. pseudotuberculosis* O:3 strains isolated around the world, some *Y. pseudotuberculosis* O:3 strains isolated in Brazil were responsible for severe and sometimes fatal diarrhea in cattle (Warth et al., 2012).

According to Warth et al. (2012), this difference in pathogenicity can be explained by the scarcity of available pastures in the winter and by the specific type of soil present in the southern region of Brazil. This type of soil is rich in iron and allows only limited vegetative growth during colder seasons. Due to the scarcity of pastures in the winter, the animals may accidentally ingest the iron-rich soil, which increases the iron concentration in the gut of reservoir animals and promotes the growth of *Y. pseudotuberculosis* strains.

The *Y. pseudotuberculosis* O:1a strains isolated in Brazil were clustered in the largest population group defined by an MLST-based population structure study (Laukkanen-Ninios et al., 2011). In addition, the *Y. pseudotuberculosis* O:1a strains deposited in the *Y. pseudotuberculosis* MLST database were typed into seven STs (9, 12, 16, 42, 43, 85, and 86) and have also been isolated worldwide from human and nonhuman sources (Fredriksson-Ahomaa, 2007; Laukkanen-Ninios et al., 2011). Unlike the *Y. pseudotuberculosis* O:3 strains, the *Y. pseudotuberculosis* O:1a strains are classified as severe gastroenteritis pathogenic strains (Fukushima et al., 2001).

The dendrogram based on the ERIC-PCR fingerprint analysis (Figure 2) grouped the 40 *Y. pseudotuberculosis* strains into two main clusters, designated ERIC-A and ERIC-B. The ERIC-A cluster was composed of three undistinguishable strains belonging to the 1/O:1a bio-serogroup. The ERIC-B cluster was composed of 37 strains belonging to the 2/O:3 bio-serogroup and was subdivided into 19 ERIC-subtypes. The differences between the ERIC patterns of the *Y. pseudotuberculosis* strains belonging to the 2/O:3 bio-serogroup were very small (85% genetic similarity), reinforcing the high genetic similarity between these strains. The isolates belonging to the ERIC-A and ERIC-B clusters exhibited a genetic similarity of 79.5%.

The fingerprint analysis based on the *Xba*I-PFGE assay (Figure 3) grouped the 40 *Y. pseudotuberculosis* strains into two major clusters, designated PFGE-A and PFGE-B. The PFGE-A cluster was composed of three undistinguishable strains belonging to the 1/O:1a bio-serogroup. The PFGE-B cluster was composed of 37 strains belonging to the 2/O:3 bio-serogroup and was subdivided into 9 PFGE subtypes. The strains belonging to the PFGE-B cluster exhibited 90.1% similarity, emphasizing their high level of genetic similarity. Isolates belonging to the PFGE-A and PFGE-B clusters had a genetic similarity of 80.1%.

The genotyping that we performed using the MLST, ERIC-PCR and PFGE assays grouped the *Y. pseudotuberculosis* strains analyzed into clusters according to their bio-serogroup (2/O:3 or 1/O:1a). A similar distribution was previously reported by Souza and Falcão (2012), who used single-nucleotide polymorphisms in the sequences of the 16S rRNA, *glnA*, *gyrB*, and *recA* genes to genotype the same *Y. pseudotuberculosis* strains used in this study. Moreover, there were good correlations between the ERIC-PCR, PFGE, and MLST clusters and the pathogenic potential of the strains tested. According to Martins and Falcão (2004) and

Martins et al. (2007), the *Y. pseudotuberculosis* strains belonging to the 1/O:1a bio-serogroup (clustered in the ST 42, ERIC-A, and PFGE-A profiles) harbor more virulence genes and are more invasive than the *Y. pseudotuberculosis* strains belonging to the 2/O:3 bio-serogroup (clustered in the ST 19, ERIC-B, and PFGE-B profiles). These authors searched for the presence of virulence genes and investigated the kinetics of infection in mice for a set of 68 *Y. pseudotuberculosis* strains isolated in Brazil, from which we selected the 40 strains that were studied in this research.

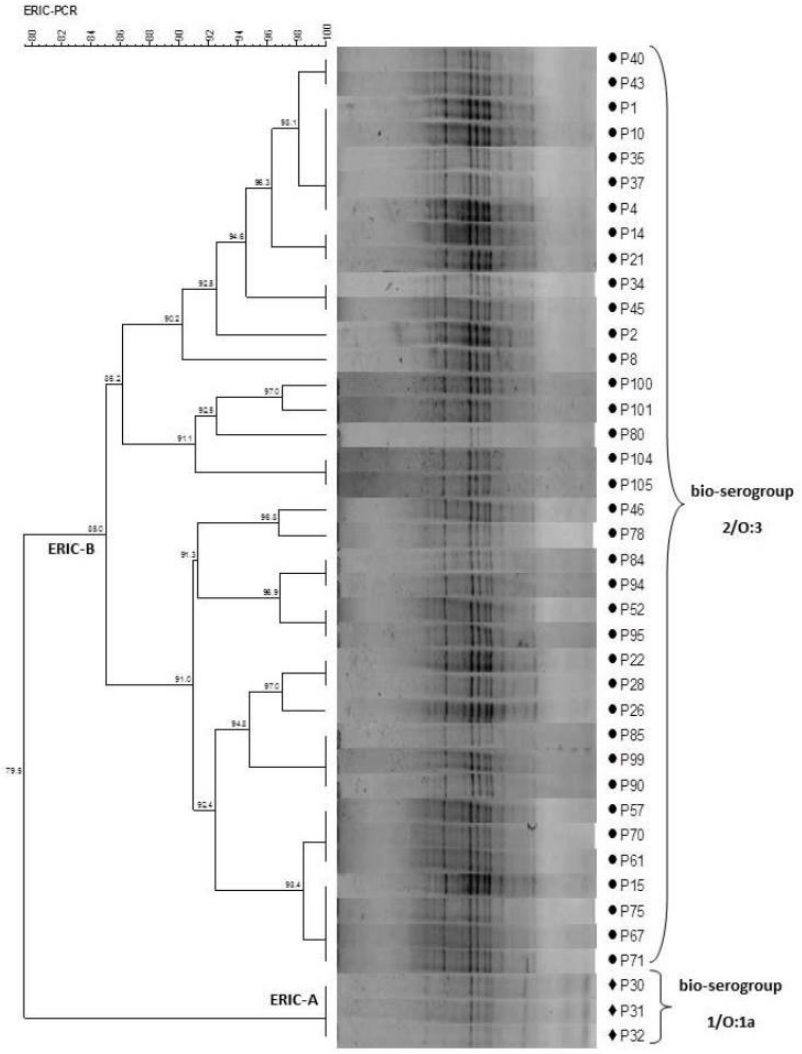

Figure 2. Phylogenetic tree based on the ERIC-PCR data showing the relationships among all 40 *Yersinia pseudotuberculosis* strains studied. Clusters were determined using the unweighted pair group method with arithmetic mean (UPGMA) method and the Dice similarity coefficient. Similarity values are shown as numbers next to nodes.

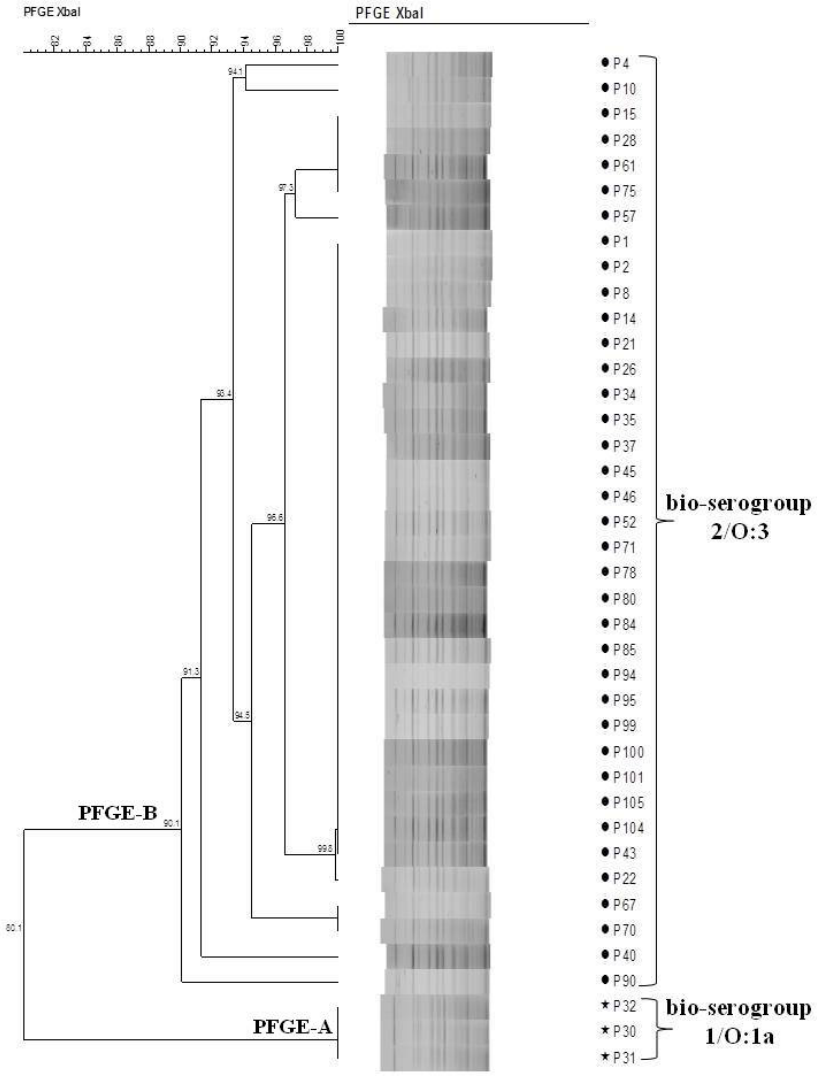

Figure 3. Phylogenetic tree based on the *Xba*I-PFGE data showing the relationships among all 40 *Yersinia pseudotuberculosis* strains studied. Clusters were determined using the unweighted pair group method with arithmetic mean (UPGMA) method and the Dice similarity coefficient. Similarity values are shown as numbers next to nodes.

No epidemiological correlation between the *Y. pseudotuberculosis* strains isolated in Brazil was observed based on their geographical origin, host and place and year of isolation, according to the ERIC-PCR, MLST, and PFGE results of this study.

It is interesting that although *Y. pseudotuberculosis* strains have never been isolated from humans in Brazil, the serogroups of the strains isolated from animals are those most often associated with *Y. pseudotuberculosis* infections in humans in other parts of the world (Fredriksson-Ahomaa, 2007; Laukkanen-Ninios et al., 2011). Also, during outbreaks of hem-

orrhagic gastroenteritis in animals, the excretion of high CFU/g feces could be responsible for environmental and food contamination, thereby spreading the infection to other animals, including humans.

As shown in this study, the MLST, ERIC-PCR, and PFGE assays were able to establish high similarity patterns among the *Y. pseudotuberculosis* strains isolated from livestock in Brazil. These three methods grouped the strains studied into clusters according to their bio-serogroup, suggesting that Brazilian livestock harbor two distinct subpopulations of *Y. pseudotuberculosis*. One subpopulation is composed of strains belonging to the 2/O:3 bio-serogroup, which is the more prevalent and diversified subpopulation, and the other subpopulation encompasses strains belonging to the 1/O:1a bio-serogroup, which is highly clonal and less common than the 2/O:3 subpopulation in Brazil.

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