Ribotyping and virulence markers of *Yersinia pseudotuberculosis* strains isolated from animals in Brazil

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Ribotyping and virulence markers has been used to investigate 68 Yersinia pseudotuberculosis *strains of serogroups O:1a and O:3. The strains were isolated from clinical material obtained from healthy and sick animals in the Southern region of Brazil. Ribotypes were identified by double digestion of extracted DNA with the restriction endonucleases* Sma*I and* Pst*I, separation by electrophoresis and hybridization with a digoxigenin-labeled cDNA probe. The presence of the chromosomal virulence marker genes* inv, irp1, irp2, psn, ybtE, ybtP-ybtQ, *and* ybtX-ybtS, *of the IS*100 *insertion sequence, and of the plasmid gene* lcrF *was detected by polymerase chain reaction. The strains were grouped into four distinct ribotypes, all of them comprising several strains. Ribotypes 1 and 4 presented distinct profiles, with 57.3% genetic similarity, ribotypes 2 and 3 presented 52.5% genetic similarity, and genetic similarity was 45% between these two groups (1/4 and 2/3). All strains possessed the* inv, irp1, *and* irp2 *genes. Additionally, strains of serogroup O:1a carried* psn, ybtE, ybtP-ybtQ, ybtX-ybtS, *and IS*100. *As expected* lcrF *was only detected in strains harboring the virulence plasmid. These data demonstrate the presence of* Y. pseudotuberculosis *strains harboring genotypic virulence markers in the livestock from Southern Brazil and that the dissemination of these bacteria may occur between herds.*

Key words: ribotyping - *Yersinia pseudotuberculosis* - virulence markers - polymerase chain reaction

Yersinia pseudotuberculosis can cause disease when it carries a set of plasmid- and chromosome-encoded virulence factors that facilitate colonization of the animal host and prevent the action of its specific and non-specific defense mechanisms (Brubaker 1991).

Virulence properties of *Y. pseudotuberculosis* strains can be studied by the analysis of virulence factors coded by genes present on a virulence plasmid (pYV), the chromosomal gene (*inv*), and the genes located on the high-pathogenicity island (HPI) (Cornelis 1994, Buchrieser et al. 1998a).

Invasiveness mediated by invasion proteins encoded by chromosomal genes is one of the main virulence mechanisms of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* species. In *Y. pseudotuberculosis*, the ability of invasion is mediated by the protein Inv coded by the chromosome gene *inv* and the protein YadA coded by the plasmid gene *yadA* (Cornelis 1994, Carniel 1995).

The capacity to capture iron by means of endogenous or exogenous siderophores is another major virulence factor. In the three pathogenic *Yersinia* species, the genes responsible for iron uptake are located in a chromosome region called the pathogenicity island. Since this region is only found in highly pathogenic *Yersinia* strains (*Y. enterocolitica* 1B, *Y. pseudotuberculosis* O:1, and *Y. pestis*), it is referred to as the HPI (Carniel 1999). The HPI of *Y. pestis* and *Y. pseudotuberculosis* comprises a core consisting of 11 genes denominated *psn*; *ybtE*, *T*, *U*, *A*, *P*, *Q*, *X*, *S*; *irp1* and *irp2*, which are involved in the biosynthesis, regulation, and transport of the siderophore yersiniabactin. The HPI also carries genes encoding the insertion element IS*100* in *Y. pestis* and *Y. pseudotuberculosis* (Bach et al. 2000). The island is inserted in the bacterial chromosome at an *asn*-tRNA locus which contains a bacteriophage P4-like attachment site at its 3'-end. Adjacent to this locus is a gene homologous to the integrase gene of bacteriophage P4 (Bach et al. 2000). The HPI of *Y. pseudotuberculosis* is serotype dependent. The complete HPI has only been found in serotype O:1. Serotype O:3 possesses an HPI that lacks a 9-kb portion on the left and carries the IS*100* sequence and genes *psn* and *ybtE*, whereas no HPI has been detected in the other serotypes tested (O:2, O:4 and O:5) (Buchrieser et al. 1998b).

Identification of the *Yersiniae* consists of biochemical and serological characterization (Aleksic & Bockemuhl 1999). These procedures are time consuming and difficult to perform and the results are obtained slowly. Polymerase chain reaction (PCR) can identify bacteria to the species level and determine whether they are virulent or not.

In the present study, we ribotyped *Y. pseudotuberculosis* strains isolated in Brazil, associating ribotypes with virulence factors present on the HPI and plasmid pYV by PCR.

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MATERIALS AND METHODS

Bacterial strains - Sixty-eight *Y. pseudotuberculosis* strains isolated from sick and healthy animals were studied. These strains belong to the Culture Collection of the *Yersinia* Reference Laboratory (Coleção de Cultura do Laboratório de Referência em Yersinia) of the São Paulo State University (Unesp) at Araraquara and were received from 1982 to 1990 for confirmation and typing. The strains originally came from the Southern region of Brazil and were isolated by various researchers from the following institutions: Marcos Enrietti Diagnostic Center, Secretariat of Agriculture of the State of Paraná, Curitiba, PR; State University of Londrina, Londrina, PR, and Federal University of Pelotas, Pelotas, RS.

Ribotyping - DNA extraction and cleavage - DNA was extracted and cleaved as described by Popovic et al. (1993) and submitted to double digestion with the enzymes *Eco*RI/*Hind*III and *Sma*I/*Pst*I. *Sma*I and *Pst*I yielded the best fingerprint profiles for differentiation of the isolates and were used for DNA cleavage in all experiments.

Probe, DNA transfer and hybridization - The 16S+23S cDNA probe was prepared by reverse transcription of 16S+23S rRNA using the DIG DNA labeling and detection kit (Boehringer Mannheim, Germany), according to manufacturer instructions. For DNA transfer and hybridization with the probe, 2 µg of DNA digest was electrophoresed on 0.8% agarose gel and transferred to nylon membranes by Southern blotting (Sambrook et al. 1989). The method used to label the 16S+23S rRNA and the detection and hybridization conditions has been described by Popovic et al. (1993).

Fingerprint analysis - Computer-assisted analysis of the ribotyping patterns was done by calculating the similarity coefficients. Cluster analysis was performed using the unweighted pair group method using arithmetic average (UPGMA). Genetic relatedness and divergence among ribotypes were calculated from a matrix defined on the basis of similarity between the distinct fingerprints (Sneath & Sokal 1973) and are represented in a dendrogram constructed with the NTSYSpc software (Exeter Software, Stony Brook, NY, 1998). Groups of isolates showing identical fingerprints were designated as ribotypes.

Determination of virulence markers and of the IS100 sequence by PCR-DNA extraction - Genomic DNA was extracted and its concentration was determined as described by Nakajima et al. (1992). Briefly, colonies from blood agar were suspended in 100 µl distilled water in one tube to achieve a concentration of 10^8 CFU/ml. The cells suspensions were boiled for 10 min to expose (denature) the DNA. Aliquots of 5 µl were then used as a template in the PCR.

Primers - The two sets of primers used in the multiplex PCR reactions targeting to *lcrF* and *inv* genes are shown in Table I. Table II shows the primers used for the detection of the HPI genes and the size (in base pairs = bp) of the expected product. *Y. pseudotuberculosis* PB1 (serotype O1a) (Fukushima et al. 2001) was used as positive control. Reactions without DNA as template were used as negative controls.

Amplification - Amplification was performed in a 50 µl reaction mixture containing 0.5 U of Taq DNA polymerase (Gibco BRL, Grand Island, NY, US), 100 µM of each deoxynucleoside triphosphate (Gibco), 0.2 µM of each primer (Gibco), and 5 µl DNA. Thirty cycles of amplification were preformed in a thermal cycler (MJ Research, Watertown, MA, US). Each cycle consisted of pre-denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 7 min.

Visualization of the amplified products - The PCR products were electrophoresed in agarose gels stained with ethidium bromide (0.5 µg/ml) and visualized by UV light.

RESULTS

Y. pseudotuberculosis ribotypes - The 68 ribotyped strains were classified into four ribotypes (R1 to R4) defined by the number (four to seven) and the size (< 2.0 to 23.1kb) of the fragments obtained by double digestion of the samples DNA with the enzymes *Sma*I and *Pst*I.

Fig. 1 shows the four different ribotype profiles detected in the 68 *Y. pseudotuberculosis* strains. Most of the strains (52.9%) belonged to ribotype 1, 20.6% to ribotype 3, 16.2% to ribotype 2, and 10.3% to ribotype 4.

Relationship among ribotypes - Percent similarity or genetic divergence among strains was calculated and is presented in the form of a dendrogram (Fig. 2). All ribotypes found included several strains. Ribotypes 1 and 4 showed different profiles and percent genetic similarity was 57.5%. Ribotypes 2 and 3 presented 52.5% genetic similarity. Genetic similarity was 45% between these two groups (1/4 and 2/3).

TABLE I

Primers used in the multiplex polymerase chain reaction for detection of the *inv* and *lcrF* genes

Target gene	Primer direction	Sequence (5'→3')	Amplicon length (bp)	Reference
inv	Forward	TAA GGG TAC TAT CGC GGC GGA	295	Nakajima et al. (1992)
	Reverse	CGT GAA ATT AAC CGT CAC ACT		
lcrF	Forward	TCA TGG CAG AAC AGC AGT CAG	591	Wren & Tabaqchali (1990)
	Reverse	ACT CAT CTT ACC ATT AAG AAG		

TABLE II

Primers used to detect the high-pathogenicity island genes *irp1*, *irp2*, *psn*, *ybtE*, *ybtP-ybtQ*, *ybtX-ybtS* and IS*100* sequence in *Yersinia pseudotuberculosis*

Target	Primer direction	Sequence (5'→3')	Amplicon length (bp)	Reference
IS*100*	Forward Reverse	ATT GAT CCA CCG TTT TAC TC CGA ACG AAA GCA TGA AAC AA	963	Podladchikova et al. (1994)
psn	Forward Reverse	CTT TCC ACC AAC ACC ATCC AAA CCG CCA CTT CGC TTC	1,062	Buchrieser et al. (1998b)
ybtE	Forward Reverse	CCC TTA CCC ATT GCC GAAC TCC CCA CCT CAT CCA GCC	1,198	Filippov et al. (1995)
irp1	Forward Reverse	AGA AAC CGA TGC TCA CCC TCC TCT CCT GAC GTA GCC	526	Filippov et al. (1995)
irp2	Forward Reverse	AAG GAT TCG CTG TTA CCG GAC TCG TCG GGC AGC GTT TCT TCT	280	Schubert et al. (1998)
ybtP-ybtQ	Forward Reverse	GCC GGG AAC GTC AAA GAA AGG TGA GCT TTC ATG TGC CT	1,816	Bach et al. (2000)
ybtX-ybtS	Forward Reverse	TCA GTC GAA TGT GAA ACC GC GCA GCC GTG CCT GGC ACC CTT T	1,453	Bach et al. (2000)

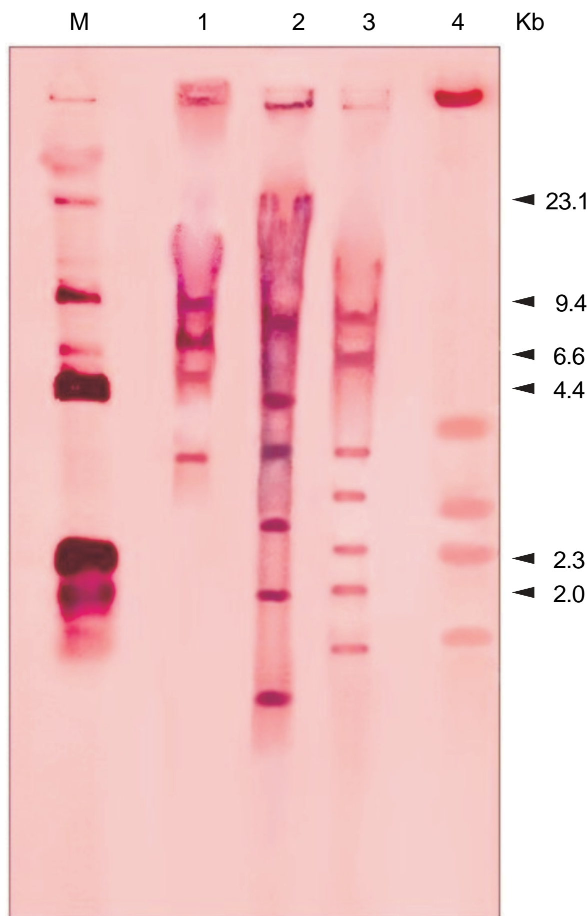

Fig. 1: ribotypes of *Yersinia pseudotuberculosis* strains observed after hybridization with digoxigenin-labeled 16+23S cDNA. Lanes - M: molecular mass marker (*Hind*III-digested λ phage DNA); 1: ribotype 1 (36 strains); 2: ribotype 2 (11 strains); 3: ribotype 3 (14 strains); 4: ribotype 4 (7 strains).

Analysis of chromosomal and plasmid genes and of sequence IS*100* - In the multiplex PCR essays the 68 strains amplified the 295-bp fragment expected for the chromosomal *inv* gene and 47 of them amplified the 591-bp fragment expected for the *lcrF* gene. These genes were detected in samples obtained from healthy and sick animals.

The other genes investigated (*psn*, *ybtE*, *ybtP-ybtQ* and *ybtX-ybtS*) and the IS*100* sequence were only detected in the two *Y. pseudotuberculosis* strains of biotype 1 and serogroup O:1a. The *inv*, *irp1,* and *irp2* genes were detected in all strains studied.

Table III shows the distribution of ribotypes among all isolates according to bioserogroup and virulence markers.

DISCUSSION

In the present study, the 68 *Y. pseudotuberculosis* strains analyzed fited into four ribotypes (Fig. 1). Ribotype 1 was the most frequent (36 strains), followed by ribotype 3, ribotype 2, and ribotype 4. Ribotype 1 and ribotype 4 showed 57.5% similarity.

No relationship was found between bioserogroups and ribotypes, although only two strains from the 1/O:1a bioserogroup were studied. These samples were classified as ribotype 1, together with 34 other strains from bioserogroup 2/O:3. In addition, no association could be established between the type of animal from which the strains were isolated and the ribotype, suggesting the circulation of *Y. pseudotuberculosis* in the environment and in different animal species. There was also no association between the ribotype and the geographical origin of the strains.

Isolation of *Y. pseudotuberculosis* cultures was carried out since 1982 in the state of Paraná and after 1989 in Rio Grande do Sul. The four ribotypes were detected in both states. These data suggest a common transmission vehicle for *Y. pseudotuberculosis* strains among animals in Paraná and Rio Grande do Sul.

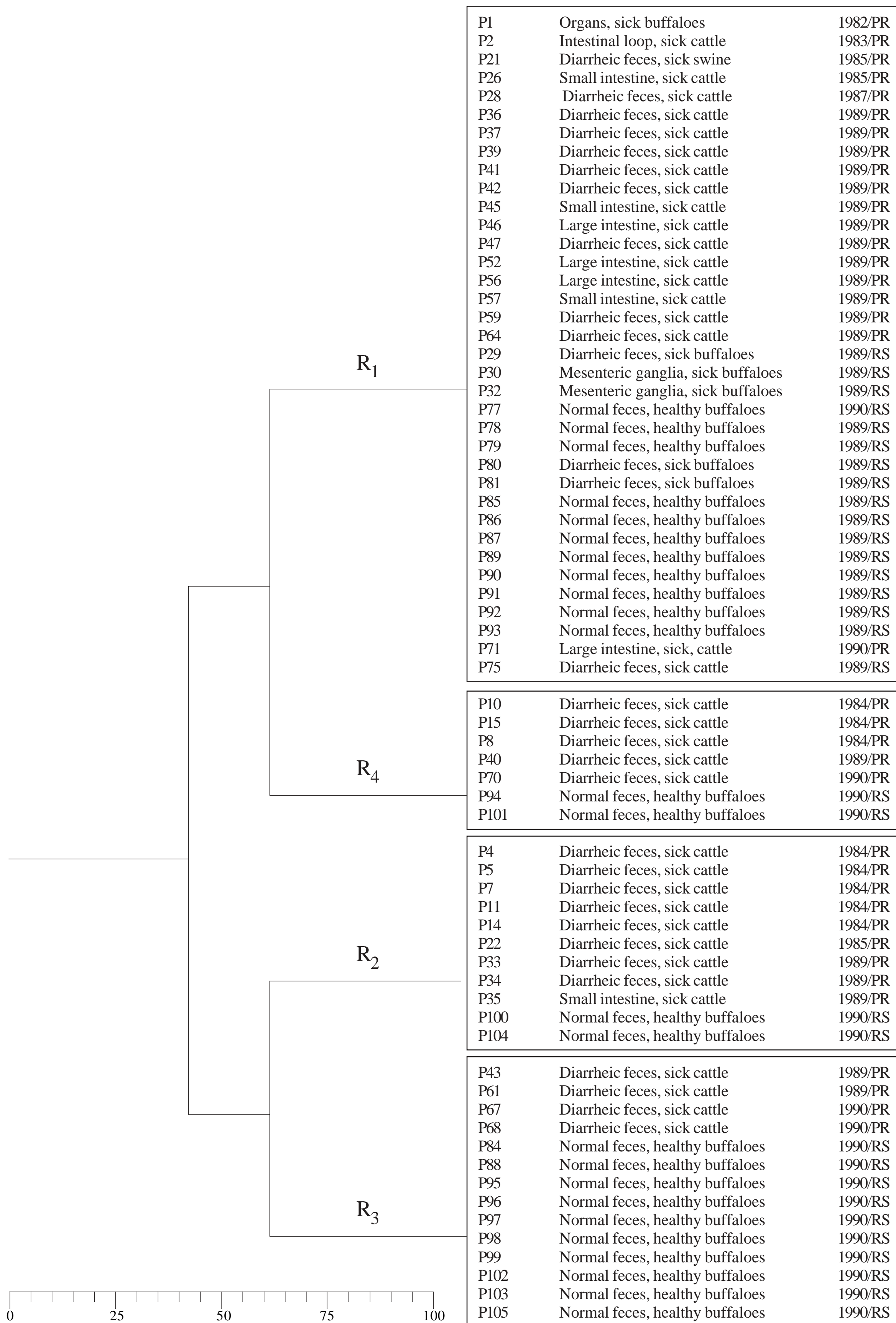

Fig. 2: dendrogram showing similarities among the ribotypes characterized for *Yersinia pseudotuberculosis* strains. The source of isolation, year and origin are shown for each strain.

TABLE III

Distribution of the four ribotypes of the *Yersinia pseudotuberculosis* strains according to bioserogroup and virulence markers

Ribotype	Strain/ bioserotype	Virulence markers
1	P1 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P2 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P21 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P26 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P28 2:O3	*inv*, *irp1*, *irp2*
	P36 2:O3	*inv*, *irp1*, *irp2*
	P37 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P39 2:O3	*inv*, *irp1*, *irp2*
	P41 2:O3	*inv*, *irp1*, *irp2*
	P42 2:O3	*inv*, *irp1*, *irp2*
	P45 2:O3	*inv*, *irp1*, *irp2*
	P46 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P47 2:O3	*inv*, *irp1*, *irp2*
	P52 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P56 2:O3	*inv*, *irp1*, *irp2*
	P57 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P59 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P64 2:O3	*inv*, *irp1*, *irp2*
	P29 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P30 1:O1a	*lcrF*, *inv*, *irp1*, *irp2*, IS*100*, *psn*, *ybtE*, *ybtP-ybtQ*, *ybtX-ybtS*
	P32 1:O1a	*lcrF*, *inv*, *irp1*, *irp2*, IS*100*, *psn*, *ybtE*, *ybtP-ybtQ*, *ybtX-ybtS*
	P77 2:O3	*inv*, *irp1*, *irp2*
	P78 2:O3	*inv*, *irp1*, *irp2*
	P79 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P80 2:O3	*inv*, *irp1*, *irp2*
	P81 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P85 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P86 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P87 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P89 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P90 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P91 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P92 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P93 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P71 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P75 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
2	P4 2:O3	*inv*, *irp1*, *irp2*
	P5 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P7 2:O3	*inv*, *irp1*, *irp2*
	P11 2:O3	*inv*, *irp1*, *irp2*
	P14 2:O3	*inv*, *irp1*, *irp2*
	P22 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P33 2:O3	*inv*, *irp1*, *irp2*
	P34 2:O3	*inv*, *irp1*, *irp2*
	P35 2:O3	*inv*, *irp1*, *irp2*
	P100 2:O3	*inv*, *irp1*, *irp2*
	P104 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
3	P43 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P61 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P67 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P68 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P84 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P88 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P95 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P96 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P97 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P98 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P99 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P102 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P103 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P105 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
4	P10 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P15 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P8 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P40 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P70 2:O3	*lcrF*, *inv*, *irp1*, *irp2*
	P94 2:O3	*inv*, *irp1*, *irp2*
	P101 2:O3	*lcrF*, *inv*, *irp1*, *irp2*

It is known, that the main sources of these microorganisms are wild rodents and birds. Contamined water can cause major outbreaks among both humans and animals, with fecal-oral transmission being the main means of acces of these bacteria (Carniel & Mollaret 1990).

Part of the *Y. pseudotuberculosis* strains studied here were isolated from healthy animals, suggesting that cattle and buffaloes may be carriers and disseminators of these bacteria. The ease transportation of animals between the two states may have also permitted the transmission of *Y. pseudotuberculosis* strains between them.

Multiplex PCR confirm the presence of the *inv* and *lcrF* genes in samples that had been previously characterized biochemically and serologically, and in which the presence of pYV had been detected in a previous study (Martins et al. 1998). These data led us to conclude that this technique is a simple and fast tool for the detection of the *inv* and *lcrF* genes (presents on the chromosome and in the pYV plasmid respectively) using primers directed to specific sequence of *Y. pseudotuberculosis*.

In this study all *Y. pseudotuberculosis* strains carried the *inv*, *irp1* and *irp2* genes. The O:3 strains carried the *inv*, *irp1* and *irp2* genes and had an incomplete HPI. This result is in agreement with published data showing that the right-hand part of the HPI contains a truncation of IS*100* sequence and the *ybtE* and *psn* genes in this serogroup (Buchrieser et al. 1998b).

The two *Y. pseudotuberculosis* O:1a strains additionally carried genes *psn*, *ybtE*, *ybtP-ybtQ*, *ybtX-ybtS,* and the IS*100* sequence, and were found to possess a complete HPI. In a previous in vivo infection kinetics study, these two samples exhibited higher pathogenicity since they were more virulent and invasive than biotype 2 and serogroup O:3 strains (Martins & Falcão 2004). Fukushima et al. (2001), studying 2235 *Y. pseudotuberculosis* strains of various bioserotypes and origins isolated from different regions of the planet, showed that all strains of the O:1a serotype exhibited a complete HPI.

The *Y. pseudotuberculosis* strains of serogroup O:3 analyzed here showed a virulence potential, since all of them carried the *irp1* and *irp2* genes belonging to the pathogenicity island, and were isolated from both sick and healthy animals. According to Carniel et al. (1992), the *irp2* gene is a marker of high pathogenicity and is only found in pathogenic strains (Almeida et al. 1993).

The absence of pYV in strains isolated from sick animals might be explained by the subsequent loss of this plasmid in some of these strains since they are stock cultures and have been frequently subcultured. On the other hand, the presence of the pathogenicity markers pYV and *irp2* gene in 30.8% of the strains isolated from healthy animals suggest a pathogenic phenotype. These animals might develop the disease under unfavorable conditions such as cold weather and food shortage and favor environmental dissemination of these strains through their feces. Another hypothesis for the healthy condition of these animals is that they possess innate resistance to infection with *Y. pseudotuberculosis* (Hodges & Carman 1985). Leal et al. (1997) phenotypically and genotypically characterizes some pathogenicity factors in *Y. enterocolitica* strains and observed the instability of some chromosomal segments in this bacterium. The authors attributed this instability to prolonged storage and manipulation after isolation, a fact indicating the importance for phenotypic and genotypic charcaterization of strains immediately after isolation.

REFERENCES

Aleksic S, Bockemuhl J 1999. *Yersinia* and other Enterobacteriaceae. In KV Forrest, JH Jorgensen, PR Murray, *Manual of Clinical Microbiology*, 7th ed., ASM Press, Washington, DC, p. 483-496.

Almeida AMP, Guiyoule A, Guilvout I, Iteman I, Baranton G, Carniel E 1993. Chromosomal *irp2* gene in *Yersinia*: distribution, expression, deletion and impact on virulence. *Microbiol Pathog 14*: 9-21.

Bach S, Almeida AMP, Carniel E 2000. The *Yersinia* high-pathogenicity island is present in different members of the family Enterobacteriaceae. *FEMS Microbiol Lett 183*: 289-294.

Brubaker RR 1991. Factors promoting acute and chronic diseases caused by Yersiniae. *Clin Microbiol Rev 4*: 309-324.

Buchrieser C, Brosh R, Bach S, Guiyoule A, Carniel E 1998a. The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn* tRNA genes. *Mol Microbiol 30*: 965-978.

Buchrieser C, Prentice M, Carniel E 1998b. The 102-kilobase unstable region of *Yersinia pestis* comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. *J Bacteriol 180*: 2321-2329.

Carniel E 1995. Chromosomal virulence factors of *Yersinia*: an update. *Contrib Microbiol Immunol 13*: 218-224.

Carniel E 1999. The *Yersinia* high-pathogenicity island. *Int Microbiol 2*: 161-167.

Carniel E, Mollaret HH 1990. Yersiniosis. *Comp Immunol Microbiol Infect Dis 13*: 51-8

Carniel E, Guiyoule A, Guilvout I, Mercereau-Puijalon O 1992. Molecular cloning, iron-regulation and mutagenesis of the *irp2* gene encoding HMWP2, a protein specific for the highly pathogenic *Yersinia*. *Mol Microbiol 6*: 379-388.

Cornelis GR 1994. *Yersinia* pathogenicity factors. *Curr Top Microbiol Immunol 192*: 243-263.

Filippov AA, Oleinikov PN, Motin VL, Protsenko OA, Sminov GB 1995. Sequencing of two *Yersinia pestis* IS elements IS285 and IS100. *Contrib Microbiol Immunol 13*: 306-309.

Fukushima H, Matsuda Y, Seki R, Tsubokura M, Takeda N, Shubin FN, Paik IK, Zheng XB 2001. Geographical heterogeneity between far eastern and western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis* strains. *J Clin Microbiol 39*: 3541-3547.

Hodges RT, Carman MG 1985. Recovery of *Yersinia pseudotuberculosis* from the faeces of healthy cattle. *N Z Vet J 33*: 175-176.

Leal, TCA, Leal NC, Almeida, AMP 1997. Marcadores de patogenicidade em *Yersinia enterocolitica* O:3 isoladas de suínos do Rio de Janeiro. *Pesq Vet Bras 17*: 19-24.

Martins CHG, Falcão DP 2004. Experimental kinetics of infection induced by *Yersinia pseudotuberculosis* isolated from stock animals. *Mem Inst Oswaldo Cruz 99*: 621-626.

Martins CHG, Bauab TM, Falcão DP 1998. Characteristics of *Yersinia pseudotuberculosis* from animals in Brazil. *J Appl Microbiol 85*: 703-707.

Nakajima H, Inoue M, Mori T, Itoh K-I, Arakawa AE, Watanabe H 1992. Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *J Clin Microbiol 30*: 2484-2486.

Podladchikova ON, Dikhanov GG, Rakin AV, Heesemann J 1994. Nucleotide sequence and structural organization of *Yersinia pestis* insertion sequence IS*100*. *FEMS Microbiol Lett 121*: 269-274.

Popovic T, Bopp CA, Olsvik ∅, Kiehlbauch JA 1993. Ribotyping in molecular epidemiology. In DH Persing, FC Tenover, TF Smith, TJ White, *Diagnostic Molecular Microbiology: Principles and Applications*, ASM Press, Washington, DC, p. 573-583.

Sambrook J, Fritsch EF, Maniatis T 1989. Analysis of genomic DNA by Southern hybridization. In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, p. 382-389.

Schubert S, Rakin A, Karch H, Carniel E, Heesemann J 1998. Prevalence of the "high-pathogenicity island" of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. *Infect Immun 66*: 480-485.

Sneath PHA, Sokal RR 1973. *Numerical Taxonomy*, WH Freeman, San Francisco, 532 pp.

Wren BW, Tabaqchali S 1990. Detection of pathogenic *Yersinia enterocolitica* by the polymerase chain reaction. *Lancet 336*: 693.