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Molecular characterization of *Corynebacterium pseudotuberculosis* isolates using ERIC-PCR

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ABSTRACT

Caseous lymphadenitis is an infectious sheep and goats disease caused by *Corynebacterium pseudotuberculosis* and characterized by abscesses in superficial and visceral lymph nodes. *C. pseudotuberculosis* strains isolated from these hosts have been shown to be very difficult to type by the existing methods. The aim of this study is evaluating the potential of the Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) as a tool for molecular typing of *C. pseudotuberculosis* strains isolated in sheep. One hundred and twenty seven isolates of *C. pseudotuberculosis* were isolated from lesions suspected to have had caseous lymphadenitis collected from sheep at the slaughterhouse. Animals were from 24 flocks in 13 municipalities of the Minas Gerais State, Brazil. Species identification of the isolates was performed by routine biochemical tests and mPCR. Fingerprint was performed by RAPD using ERIC-1R, ERIC-2 and ERIC-1R + ERIC-2 primers. Seventeen different genotypes were generated by ERIC 1-PCR, 21 genotypes by ERIC 2-PCR and 21 genotypes by ERIC 1 + 2-PCR. Hunter-Gaston Discrimination Index (HGDI) found for ERIC 1, ERIC 2, ERIC 1 + 2 PCR were 0.69, 0.87, and 0.84, respectively. For most herds evaluated observed at most three different genotypes among isolates from animals of these property, in all ERIC-PCR assays. However a few flocks observed between four and nine genotypes per flock. The W Kendall value found for correlation among the three techniques of ERIC-PCR was 0.91 ($P < 5.01 \times 10^{-6}$). The results show that ERIC-PCR has good discriminatory power and advantages over other DNA-based typing methods, making it a useful tool to discriminate *C. pseudotuberculosis* isolates.

1. Introduction

Corynebacterium pseudotuberculosis is a relevant animal pathogen belonging to the family of Corynebacteriaceae classified into two biotypes based on hosts preferences and nitrate-reducing activity (Biberstein et al., 1971). In sheep, it is responsible for caseous lymphadenitis characterized by abscesses in the superficial and visceral lymph nodes and is widely disseminated among the sheep population in

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Minas Gerais State, Brazil, with 75.8% of real prevalence (Guimarães et al., 2009).

Several molecular techniques have been proposed to type *C. pseudotuberculosis*, as Restriction Fragment Length polymorphism (RFLP) (Songer et al., 1988; Sutherland et al., 1996; Bjorkroth et al., 1999), Ribotype (Sutherland et al., 1993), Pulse-Field Gel Eletrophoresis (PFGE) (Connor et al., 2000, 2007) and Random Amplified Polymorphic DNA (RAPD) (Foley et al., 2004; Stefańska et al., 2008). Connor et al. (2007) using PFGE for typing 47 *C. pseudotuberculosis* isolates from small ruminants and horse from various countries, classified the strains into four pulsotypes and concluded that genome of *C. pseudotuberculosis* is highly conserved, irrespective of the country of strain origin. Foley et al. (2004), working with RAPD-PCR and strains of *C. pseudotuberculosis* isolates from horses, cattle and sheep from states of Colorado, Kentucky, Utah and California, found 10 different genotypes.

In this context, the Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), a simple technique of random amplified polymorphic DNA, has been successfully applied in genotyping of microbial pathogens, including gene mapping, detection of strain diversity, population analysis, epidemiology, and the demonstration of phylogenetic and taxonomic relationships (Li et al., 2009). All isolates are typeable and no prior knowledge of target genome sequences is necessary (Maslow and Mulligan, 1996; Stefańska et al., 2008).

Thus, the aim of this study was evaluating the potential of ERIC-PCR as a tool for molecular typing of strains of *C. pseudotuberculosis* isolated from sheep in Minas Gerais State, Brazil as well as establishing a protocol.

2. Materials and methods

2.1. Bacterial strains and culture conditions

One hundred and twenty seven strains of *C. pseudotuberculosis* were studied derived from 24 herds from 13 municipalities. All strains were isolated from lesions suspected to caseous lymphadenitis collected in slaughterhouse with Federal Inspection Service, located in Patrocínio municipality, Minas Gerais State, Brazil. The purulent material was collected between July and December of 2007, every 15 days, by two veterinarians.

The state of Minas Gerais is located in southeastern Brazil. It has a geographical area of 588,383 km^2, typical tropical climate, and a mean annual temperature of 21.2 °C. Annual rainfall varies from 1000 to 2000 mm, with well-defined dry and wet seasons.

The isolates were grown on agar base (Difco, USA) added of 5% defibrinated sheep blood and incubated at 37 °C for 48 h. They were identified by standard biochemical tests (MacFaddin, 1980) and characterized according Coyle et al. (1985). Nitrate reduction was confirmed using nitrate broth and further reduction beyond nitrite was tested by the addition of zinc dust. Specie identification was also determined using mPCR (Pacheco et al., 2007).

2.2. Isolation of DNA

The genomic DNA was extracted from the *C. pseudotuberculosis* cells as previously described by Pitcher et al. (1989). DNA quality and concentration were determined by spectrophotometry (Sambrook and Russell, 2001).

2.3. ERIC-PCR conditions

The strains of *C. pseudotuberculosis* identified were fingerprinted by RAPD using the primers ERIC-1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) and ERIC-1R + ERIC-2 primer pair (Invitrogen, USA) (Versalovic et al., 1991). Optimal reaction mixture was determined as 25.0 μl containing 1X buffer (50.0 mM Tris, 1.5 mM $MgCl_2$, 10.0 mM KCl, 50.0 mM $(NH4)_2SO_4$, pH 8.3) (Phoneutria, Brazil); 0.2 mM of each (dNTP) (Invitrogen, USA); 2.0 μM of each primer (Invitrogen, USA); 2.5 units of Taq DNA polymerase (Phoneutria, Brazil) and 100.0 ng of DNA. Thermocycling conditions (Hybaid Thermal Cycler, Thermo Scientific, USA) were as follow: initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 1 min, and elongation at 72 °C for 2 min. The final extension step was performed at 72 °C for 5 min. The *C. pseudotuberculosis* ATCC 19410 was used as positive control and deionized water was the negative control, both were added in all experiments. The amplified products were submitted to electrophoresis in 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer (89 mM Tris Base, 89 mM Boric Acid and 2 mM EDTA pH8.0), at 110 V for 1.5 h, stained with 0.5 mg/ml ethidium bromide, visualized under UV light, and photographed (Image-Master VDS, Phamarcia Biotech, Sweden). The DNA molecular weight marker used was a 1 Kb Plus DNA Ladder (Invitrogen, USA). To avoid interpretation troubles associated with interassay variability, all the strains were subjected to each assay at the same time and in similar conditions, only one electrophoresis was made for each primer or primer pair evaluated.

2.4. Genotype analysis

Band size estimates and genotype analyses were done using the software BioNumerics 5.1 (Applied Maths, Belgium). Clustering analysis was performed with the same software based on the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA). The Hunter and Gaston Diversity Index (HGDI) were calculated according to Hunter and Gaston (1988) for ERIC 1, ERIC 2, and ERIC 1 + 2.

2.5. Repeatability

To assess the repeatability of ERIC-PCR *C. pseudotuberculosis* reference strain (ATCC 19410), *C. pseudotuberculosis* vaccine strain 1002 and four *C. pseudotuberculosis* field isolated were submitted to five different amplifications assays for each primer or primer pair tested. The assays were realized in similar conditions as described in section ERIC-PCR conditions, in different days for each primer or

primer pair. The results obtained were analyzed using the software BioNumerics 5.1 (Applied Maths, Belgium).

2.6. Statistic analysis

The global agreement between the three techniques was calculated evaluating the number (n) of different genotypes in each assay of ERIC-PCR per farm sheep supplier using the nonparametric Kendall's W statistic (Matthias et al., 2009) with the aid of R software version 2.9.0 (R Development Core Team, 2009).

3. Results

3.1. ERIC genotypes

One hundred and twenty seven strains of *C. pseudotuberculosis* isolates from sheep, *C. pseudotuberculosis* 1002 vaccine strain and *C. pseudotuberculosis* (ATCC 19410) reference strain were fingerprinted by ERIC-PCR and all strains compared were always included in a single run in order to avoid misinterpretation problems associated with interassay variability. Adopting 100% of similarity, 17 genotypes were obtained for ERIC 1-PCR and sequentially named E1.1 to E.1.17 (Fig. 1), 21 genotypes for ERIC 2-PCR, named E2.1 to E2.21 (Fig. 2) and 21 genotypes for ERIC 1 + 2-PCR named 12.1 to E12.21 (Fig. 3).

For ERIC 1-PCR, the E1.7 genotype was the most prevalent containing 48.8% (63/129) of strains, the E1.6 occurred in 25.6% (33/129) of strains and both genotypes together covered 74.4% of total strains studied. The others strains were distributed among the 15 different genotypes remaining. Three fragments were more frequent in this ERIC-PCR, present in 93.8% of total strains, being approximately 174 bp, 343 bp and 585 bp (Fig. 1).

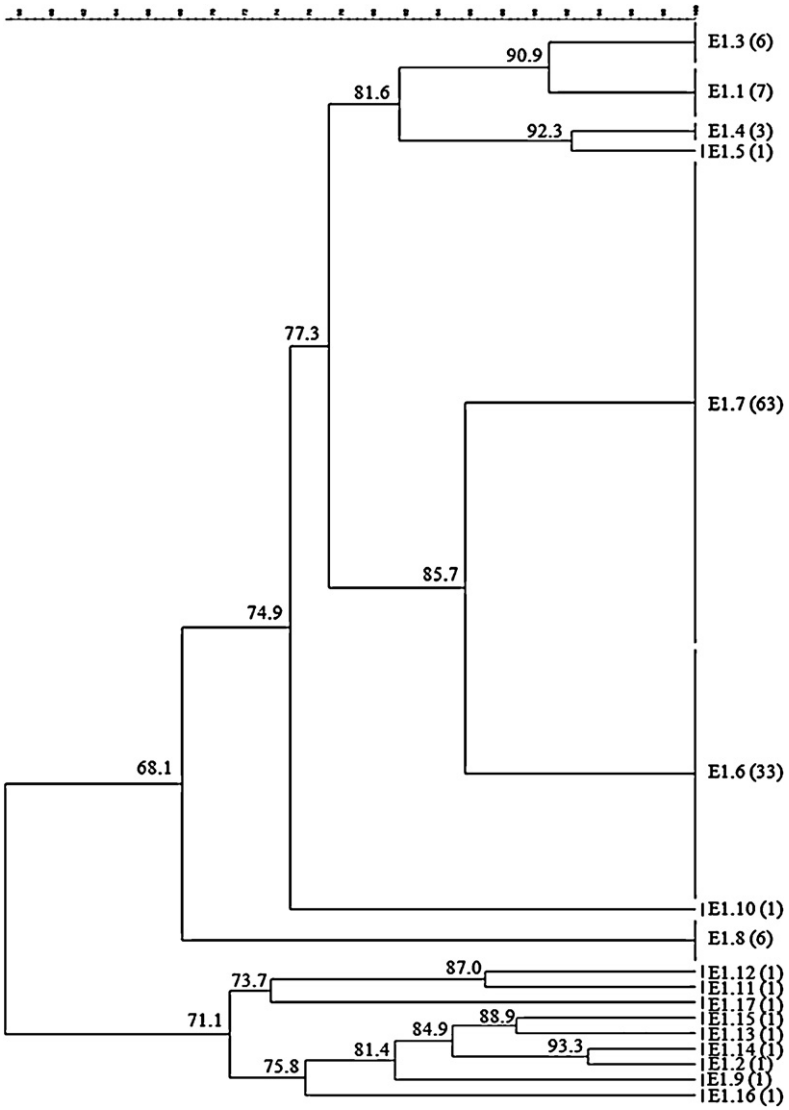

Fig. 1. Cluster analysis by ERIC-PCR fingerprint (ERIC 1) of 127 *C. pseudotuberculosis* isolates from sheep in Minas Gerais Sate, 1002 *C. pseudotuberculosis* vaccine strain and 19410 (ATCC) *C. pseudotuberculosis* reference strain.

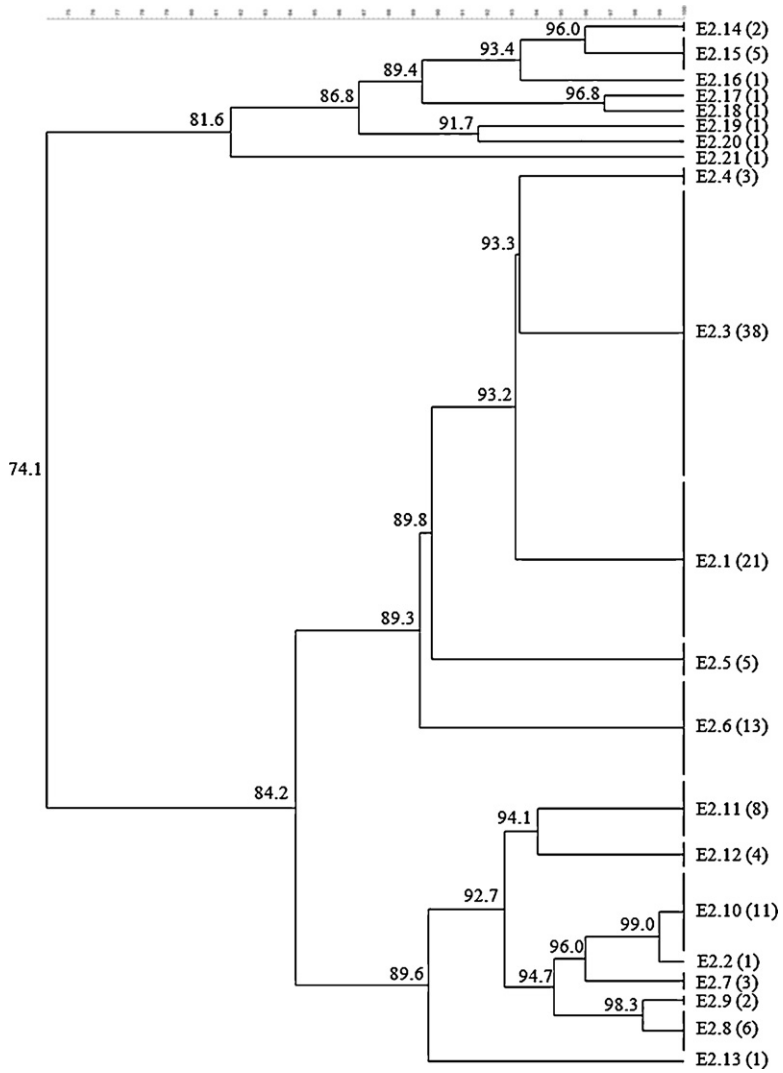

Fig. 2. Cluster analysis by ERIC-PCR fingerprint (ERIC 2) of 127 *C. pseudotuberculosis* isolates from sheep in Minas Gerais State, 1002 *C. pseudotuberculosis* vaccine strain and 19410 (ATCC) *C. pseudotuberculosis* reference strain.

For ERIC 2-PCR, the E2.3 genotype was the most prevalent containing 29.5% (38/129) of strains, E2.10 genotype occurred in 16.3% (21/129) and both genotypes together covered 45,8% of total strains studied. The others strains are distributed among 19 different genotypes remaining. Three fragments were more frequent in this assay, approximately 205 bp, 374 bp and 425 bp, present in 99.2% of strains (Fig. 2).

For ERIC 1 + 2-PCR, the E12.9 DNA pattern was the most prevalent containing 32.6% (42/129), the E12.14 occurred in 15.5% (20/129) and the E12.11 covered 13.2% (17/129) of the strains studied. All these genotypes together covered 61.3% of total strains studied. The others strains are distributed among 18 different genotypes remaining. Four fragments were more frequents in this assay, with approximately 109 bp, 171 bp, 399 bp and 630 bp, present in 96.9% of strains (Fig. 3).

ERIC-PCR did not show molecular difference between *C. pseudotuberculosis* nitrate-positive and nitrate-negative strains of for neither assay. Fig. 4 shows the ERIC-PCR profiles isolates of *C. pseudotuberculosis.*

3.2. Repeatability

The repeatability found for the three techniques were 85.7%, 90.9% and 88.9% for ERIC 1-PCR, ERIC 2-PCR and ERIC 1 + 2-PCR, respectively. These variations were found in six different strains tested in five experiments performed on different days for the three techniques (Data not shown).

3.3. Discriminatory index

The HGDI calculated for the ERIC 1-PCR was 0.69, for the ERIC 2-PCR was 0.87 and for the ERIC 1 + 2-PCR was 0.84.

3.4. Techniques agreement

The W Kendall value found for correlation among the three techniques of ERIC-PCR was 0.91 ($P < 5.01 \times 10^{-6}$), since 1 mean perfect correlation and 0 means no correlation.

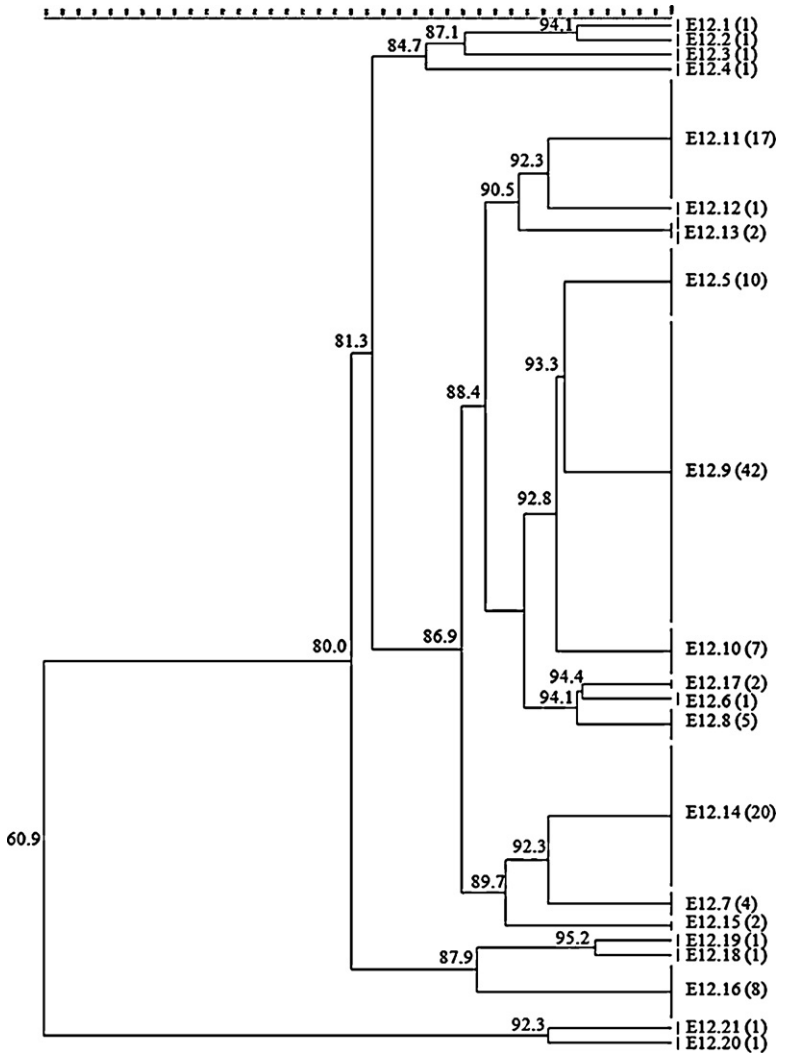

Fig. 3. Cluster analysis by ERIC-PCR fingerprint (ERIC 1 + 2) of 127 *C. pseudotuberculosis* isolates from sheep in Minas Gerais State, 1002 *C. pseudotuberculosis* vaccine strain and 19410 (ATCC) *C. pseudotuberculosis* reference strain.

3.5. Geographic distribution

The most of the herds showed three different genotypes for all ERIC-PCR assays evaluated. The results are summarized in Table 1.

4. Discussion

The molecular characterization of the *C. pseudotuberculois* achieved through several techniques (PFGE, RFLP, RAPD) have shown high genetic homogeneity within species (Sutherland et al., 1993, 1996; Foley et al., 2004; Connor et al., 2007). In contrast, ERIC-PCR assay allowed good genetic differentiation for *C. pseudotuberculosis* strains, resulting in 17 or 21 distinct genotypes dependent of the primer used and high discriminatory power index (HGDI) for all PCR assays tested. The HGDI is a key characteristic of typing systems and is based on an estimate of its ability to differentiate between two unrelated strains (Hunter, 1990). The high HGDI found for three techniques studied is even more evident when compared to other typing techniques already employed for the genus *Corynebacterium*.

The greatest discriminatory power and typeability observed to the ERIC-PCR could be explained by large number of isolates typed, all the techniques used for typing of *C. pseudotuberculosis* reported in the literature use a low number of isolates 6 (Sutherland et al., 1993), 13 (Sutherland et al., 1996) and 47 (Connor et al., 2007). The inclusion of a large collection of strains and different typing markers is helpful to assess the discriminatory index of method and linking genotypes with phenotypical and epidemiological traits (Li et al., 2009). Regarding the association of genotypes with phenotypic characteristics, genetic differences between nitrate-positive and nitrate-negative *C. pseudotuberculis* strains were not observed for ERIC-PCR, despite has already been detected by RFLP (Sutherland et al., 1996). However, direct comparison

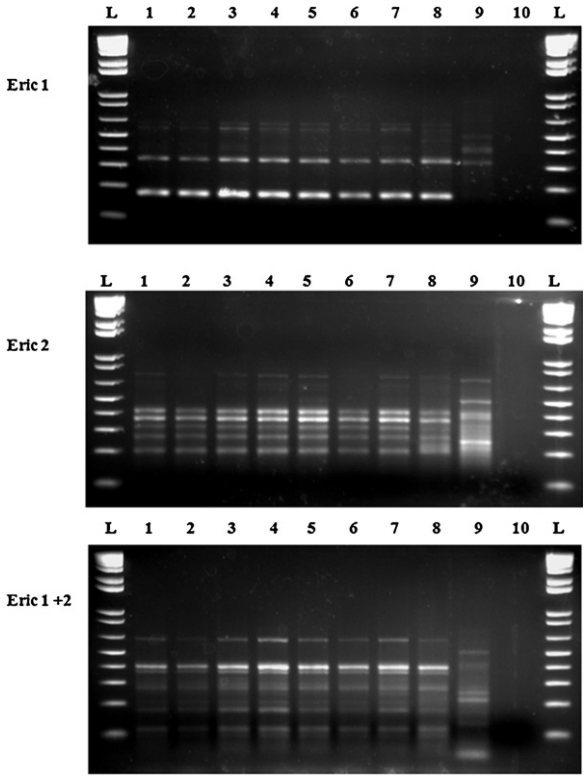

Fig. 4. ERIC 1, ERIC 2, ERIC 1 + 2 profiles of *C. pseudotuberculosis* and *Rhodococcus equi*. Lanes 1–6, field ovine isolates, lane 7, 1002 *C. pseudotuberculosis* vaccine strain; lane 8, *C. pseudotuberculosis* ATCC 19410, Lane 9, *Rhodococcus equi*; Lane 10, negative control, L, 1 Kb plus DNA Ladder (Invitrogen, USA).

among different techniques of typing is difficult because they may be based on cleavage of DNA using restriction enzymes (RFLP, PFGE), DNA hybridization (Ribotyping) or random amplifications (RAPD) and polymorphisms analyzed in each of these methods are subject to different evolutionary mechanisms (Tibayrenc, 1998). The application of bacterial typing tools requires understanding of both the strengths and limitations of the chosen bacterial (Foxman et al., 2005).

PCR methods for molecular typing require careful in-house validation of reproducibility, repeatability and discrimination (Struelens, 2002). ERIC-PCR is an inexpensive, sensitive, and fast, tool for molecular typing which requires no prior knowledge of the genome (Foxman et al., 2005; Li et al., 2009). However, the repeatability and reproducibility of this method remains a challenge, which hinders comparison of ERIC-PCR patterns between and within laboratories (Power, 1996; Tyler et al., 1997). In this study, repeatability above 85% was obtained for three techniques; being an index that can be considered very good, especially for techniques based on RAPD and although the reproducibility has not been assessed directly the results found for repeatability suggesting that this technique could be exported among laboratories. In spite of having a 10% variation among tests of ERIC-PCR, a cutoff of 100% was adopted for the analysis of all isolates in this study, since all strains were genotyped in a single experiment for each primer or primer pair. Moreover, the W Kendall value found in the evaluation of agreement among the ERIC-PCR shows that regardless of technique used (ERIC 1, ERIC 2 or ERIC 1 + 2) for genotyping of isolates of *C. pseudotuberculosis* the result is very similar. Thus, it is possible to choose any one of the individual technique for genotyping the species.

The Brazilian breed Santa Inês constitutes majority of the Minas Gerais sheep population, which was initially found in the northeastern regions of Brazil (ARCO, 2009). Recent data (2008/2009) of the state government agency for animal health (Instituto Mineiro de Agropecuária - IMA) shows that there was a large traffic of sheep from different states of Brazil (Distrito Federal, Espírito Santo, Goiás, Rio de Janeiro, Sergipe, São Paulo) contributing to the constant herd population growth in Minas Gerais. The growth of Brazilian sheep herds is focused on meat production, from the cross breeding of imported fleece animals from various countries, mainly South Africa and Europe, with woodless animals from various regions of Brazil, especially the Northeast. These growth data from the national sheep flock and especially from Minas Gerais justify the results showing that majority of the properties presented up to three different genotypes within the herd (Table 1) with similar percentages in the assays, and for the others herds the number of genotypes showed varied genotypes within four and nine. Great numbers of different genotypes found in the same herd is probably due to growth of 54.9% sheep herd in Minas Gerais, much higher than the national average that was 14.2%, between 2003 and 2008 (IBGE, 2010), with intense traffic of animals sold for slaughter or breeding for arrays and formation of new herds. Thus, the absence of specific national health legislation for the sheep allowed the introduction of exotic diseases so far in national breeding stocks and their spread to other regions of the country (ACCOMIG, 2010). Moreover, Minas Gerais State has a particular characteristics is composed mainly by small and medium herds, and because of these, many farmers

Table 1
Number (*n*) of different genotypes found in sheep supling herds to slaughterhouse in Minas Gerais State, Brazil, in each assay ERIC-PCR.

Number (*n*) of Genotypes	ERIC 1 Number (*n*) of herds	%	ERIC 2 Number (*n*) of herds	%	ERIC 1 + 2 Number (*n*) of herds	%
1	5	20.9	5	20.9	5	20.9
2	9	37.6	10	41.8	8	33.0
3	5	20.9	1	4.1	2	8.3
4–9	5	20.6	8	33.2	9	37.8
Total	24	100.0	24	100.0	24	100.0

have associated themselves with the objective of buying, input, and selling the animals. These aspects consequently promoted further contact between animals from different herds. The large dynamic found in sheep flocks from Minas Gerais State associated to its recent formation and with the introduction of animals from different regions may have contributed to the large number of genotypes observed in a few some properties studied.

Additionally, *C. pseudotuberculosis* can survive for up to six months in the environment and caseous lymphadenitis is a long term disease, beyond the infected animal remains in this condition for its life span, disseminating the agent through purulent discharge of lymph nodes (O'Reilly et al., 2008). All these factors associated the lack of control measures done by farms and with deficient diagnosis and vaccination collaborate for the dissemination of this infectious agent throughout the production network (Guimarães et al., 2009). The live vaccine 1002 strain, produced by Empresa Baiana de Desenvolvimento Agrícola em 2000 (EMBRAPA, 2000), showed similar profile of most of the strains tested, confirming that the vaccine strain is genetically related with field strains. All herds sampled not used this vaccine strain (1002), so the genotypes analyzed in this study are only from field strains. The knowledge of genotypic profile of the Brazilian strains can contribute for production of more effective vaccines.

5. Conclusion

The protocol established in this study proved to be simple, inexpensive and useful to discriminate several patterns of *C. pseudotuberculosis*, and indicates that ERIC-PCR offers some advantages over other DNA-based typing methods. It is a faster and technically simple method which has the highest discriminatory power allowing study of distribution of a single type in different individuals. The results of this study were particularly important to understand epidemiology and to control the *C. pseudotuberculosis* infection.

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