GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Short Communication

Molecular characterization of *Corynebacterium pseudotuberculosis* isolated from goats using ERIC-PCR

E.M.S. Dorneles[1], J.A. Santana[1], G.I. Andrade[1], E.L.S. Santos[1], A.S. Guimarães[2,3], R.A. Mota[4], A.S. Santos[4], A. Miyoshi[3,5], V. Azevedo[3,5], A.M.G. Gouveia[1,3], A.P. Lage[1,3]* and M.B. Heinemann[1,3]*

[1]Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[2]Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Leite, Juiz de Fora, MG, Brasil
[3]Grupo de Extensão da Pesquisa em Ovinos e Caprinos, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[4]Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Recife, PE, Brasil
[5]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

*These authors contributed equally to the study.
Corresponding author: M.B. Heinemann
E-mail: mabryan@ufmg.br

Genet. Mol. Res. 11 (3): 2051-2059 (2012)
Received November 10, 2011
Accepted March 13, 2012
Published August 6, 2012
DOI http://dx.doi.org/10.4238/2012.August.6.9

ABSTRACT. *Corynebacterium pseudotuberculosis*, the infectious agent of caseous lymphadenitis (CLA), is responsible for substantial economic losses in goat and sheep production. Molecular characterization of *C. pseudotuberculosis* isolates by enterobacterial repetitive intergenic consensus (ERIC)-PCR has shown promising results in genotyping strains isolated from sheep with CLA. We evaluated the genetic diversity of *C. pseudotuberculosis* isolates collected from the Sertão

region of the Pernambuco (PE) State, Brazil, and investigated the potential of ERIC-PCR as a tool for the molecular typing of strains of *C. pseudotuberculosis* isolated from goats. Thirty-two *C. pseudotuberculosis* strains isolated from goats in the municipalities of Floresta and Ibimirim, PE, *C. pseudotuberculosis* type strain ATCC 19410, the 1002 vaccine strain, and a field isolate of *Rhodococcus equi* were fingerprinted using the primers ERIC-1R and ERIC-2 and the primer pair ERIC-1R+ERIC-2. Using 100% similarity as the cutoff, 8, 10, and 7 genotypes were obtained with ERIC-1-PCR, ERIC-2-PCR, and ERIC-1+2-PCR, respectively. The Hunter-Gaston discriminatory index calculated for the ERIC-1-PCR was 0.75. The index for the ERIC-2-PCR was 0.88, and the index for the ERIC-1+2-PCR was 0.79. Among goat isolates of *C. pseudotuberculosis*, three, two and four genotypes (found by ERIC-1-PCR, ERIC-2-PCR, and ERIC-1+2-PCR, respectively) had been previously described among sheep isolates from Minas Gerais State, Brazil. These results showed that ERIC-PCR has good discriminatory power and typeability, making it a useful tool for discrimination among *C. pseudotuberculosis* isolates from goats.

Key words: *Corynebacterium pseudotuberculosis*; ERIC-PCR; Caseous lymphadenitis; Goats

INTRODUCTION

Corynebacterium pseudotuberculosis is a Gram-positive, non-spore forming, facultative intracellular bacterium classified into 2 biotypes based on host preferences and nitrate-reducing activity (Baird and Fontaine, 2007; Guimarães et al., 2011a). *C. pseudotuberculosis* is the etiological agent of caseous lymphadenitis (CLA) in goats and sheep (Guimarães et al., 2011a), which is characterized by abscesses in superficial and visceral lymph nodes and is associated with significant losses in animal production (Baird and Fontaine, 2007). CLA is distributed worldwide and has a major impact in most sheep- and goat-raising areas (Baird and Fontaine, 2007; Seyffert et al., 2010, Guimarães et al., 2009, 2011a).

The molecular characterization of *C. pseudotuberculosis*, achieved through restriction fragment length polymorphism (Sutherland et al., 1996), ribotyping (Sutherland et al., 1993), pulse-field gel electrophoresis (Connor et al., 2000), and random amplified polymorphic DNA (RAPD; Foley et al., 2004) analyses, has revealed high genetic homogeneity within the species. Subtyping of *C. pseudotuberculosis* strains isolated from various hosts using existing genotyping methods has proven to be difficult. Recently, we proposed a typing method based on enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR), which we found to be an excellent test for the genetic discrimination of *C. pseudotuberculosis* strains from sheep and had high resolution and good repeatability (Guimarães et al., 2011b). Thus, the aims of this study were to evaluate the genetic diversity of *C. pseudotuberculosis* isolates in the Sertão Region of Pernambuco State, Brazil, using ERIC-PCR and to investigate the potential use of this technique as a tool for molecular typing of *C. pseudotuberculosis* strains isolated from goats.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Thirty-four *C. pseudotuberculosis* strains, including 32 field isolates from goats, the 1002 vaccine strain, the ATCC 19410^T type strain, and 1 *Rhodococcus equi* field isolate, were used in this study. All *C. pseudotuberculosis* field strains were isolated from suspected CLA lesions and were collected from goats in the municipalities of Floresta (7) and Ibimirim (25) in the Sertão Region of the Pernambuco State, Brazil. The purulent material was collected between 2008 and 2009.

The isolates were plated on agar (Difco, USA) with 5% defibrinated sheep blood and incubated at 37°C for 48 h. They were identified using standard biochemical tests (MacFaddin, 1980) and characterized according to the methods of Coyle et al. (1985). Species identification was also confirmed using phospholipase D PCR (Pacheco et al., 2007).

Genomic DNA extraction

Genomic DNA was extracted from *C. pseudotuberculosis* cells according to the methods of Pitcher et al. (1989).

ERIC-PCR conditions

C. pseudotuberculosis isolates were fingerprinted with RAPD using the following primers (Versalovic et al., 1991) as described by Guimarães et al. (2011b): ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3'), ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGC G-3'), and the ERIC-1R+ERIC-2 primer pair (Invitrogen, USA). To avoid misinterpretation owing to interassay variability, all strains were assayed at the same time and under similar conditions. Only 1 electrophoresis was performed for each primer or primer pair evaluated.

Genotype analysis

Band size estimates and genotype analysis were performed using BioNumerics 6.0 (Applied Maths, Belgium). Clustering analysis was performed based on the Dice coefficient and the unweighted pair-group method with arithmetic mean using the same software. The Hunter-Gaston diversity index (HGDI) was calculated to evaluate the discriminatory power of all ERIC-PCR tests (Hunter and Gaston, 1988). Typeability was evaluated from the proportion of isolates that were scored in the ERIC-PCR assays and assigned a type (Struelens, 1998).

RESULTS

The molecular characterization of *C. pseudotuberculosis* isolates with ERIC-PCR using 100% similarity as a cutoff point revealed 8 genotypes through ERIC-1-PCR (Figure 1), 10 genotypes through ERIC-2-PCR (Figure 2), and 7 genotypes through ERIC-1+2-PCR (Figure 3). The HGDIs calculated for the ERIC-1-PCR, ERIC-2-PCR, and ERIC-1+2-PCR were 0.7496, 0.8824, and 0.7933, respectively. In the typeability test, all *C. pseudotuberculosis* and *R. equi* strains tested

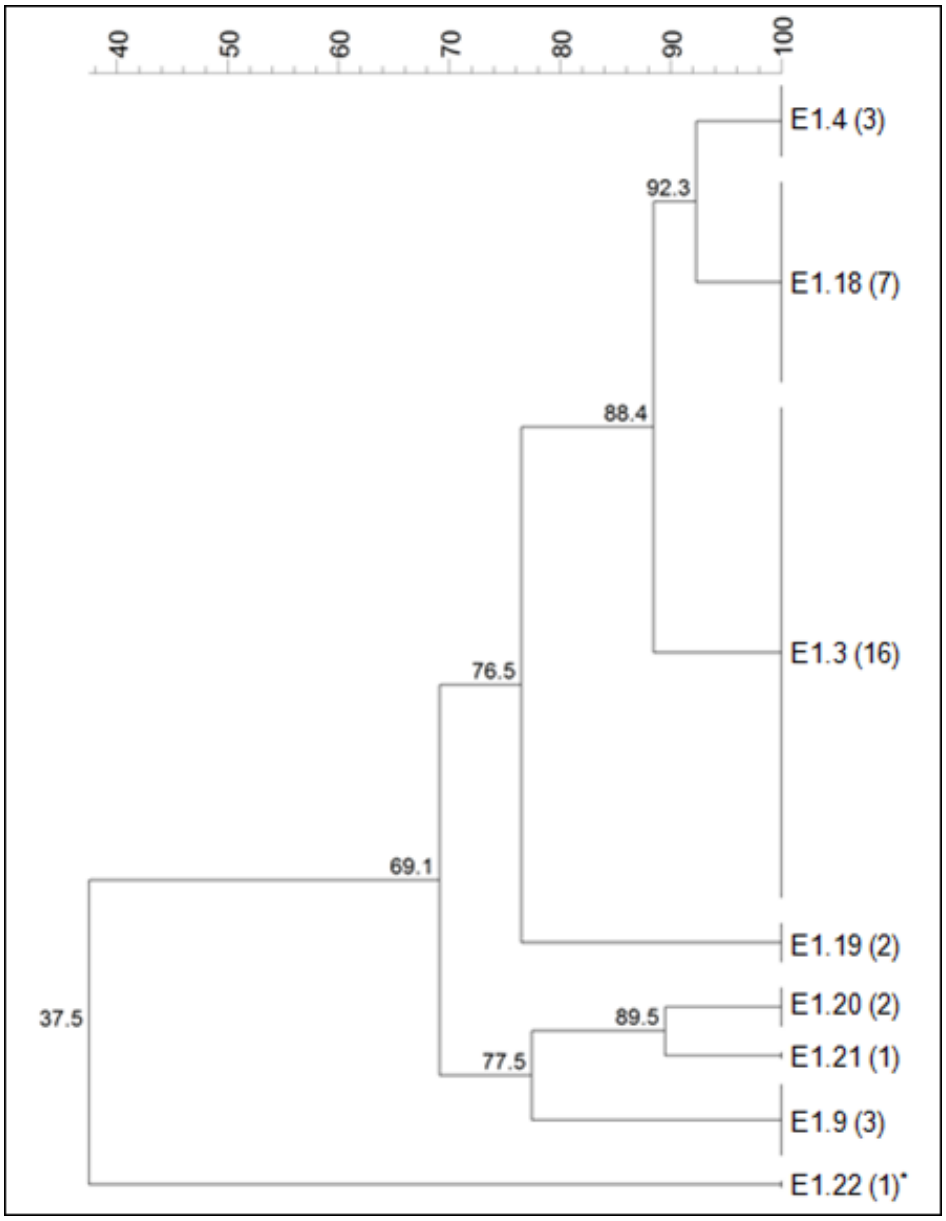

Figure 1. Cluster analysis of the ERIC-PCR fingerprint (ERIC-1) of 32 *Corynebacterium pseudotuberculosis* isolates from goats in the Sertão Region of the Pernambuco State, Brazil. The *C. pseudotuberculosis* type strain ATCC 19410^{T}, the 1002 vaccine strain, and a field isolate of *Rhodococcus equi* were also included. The numbers in parentheses correspond to the number of isolates within the genotype. The field isolated of *R. equi* is denoted by an asterisk.

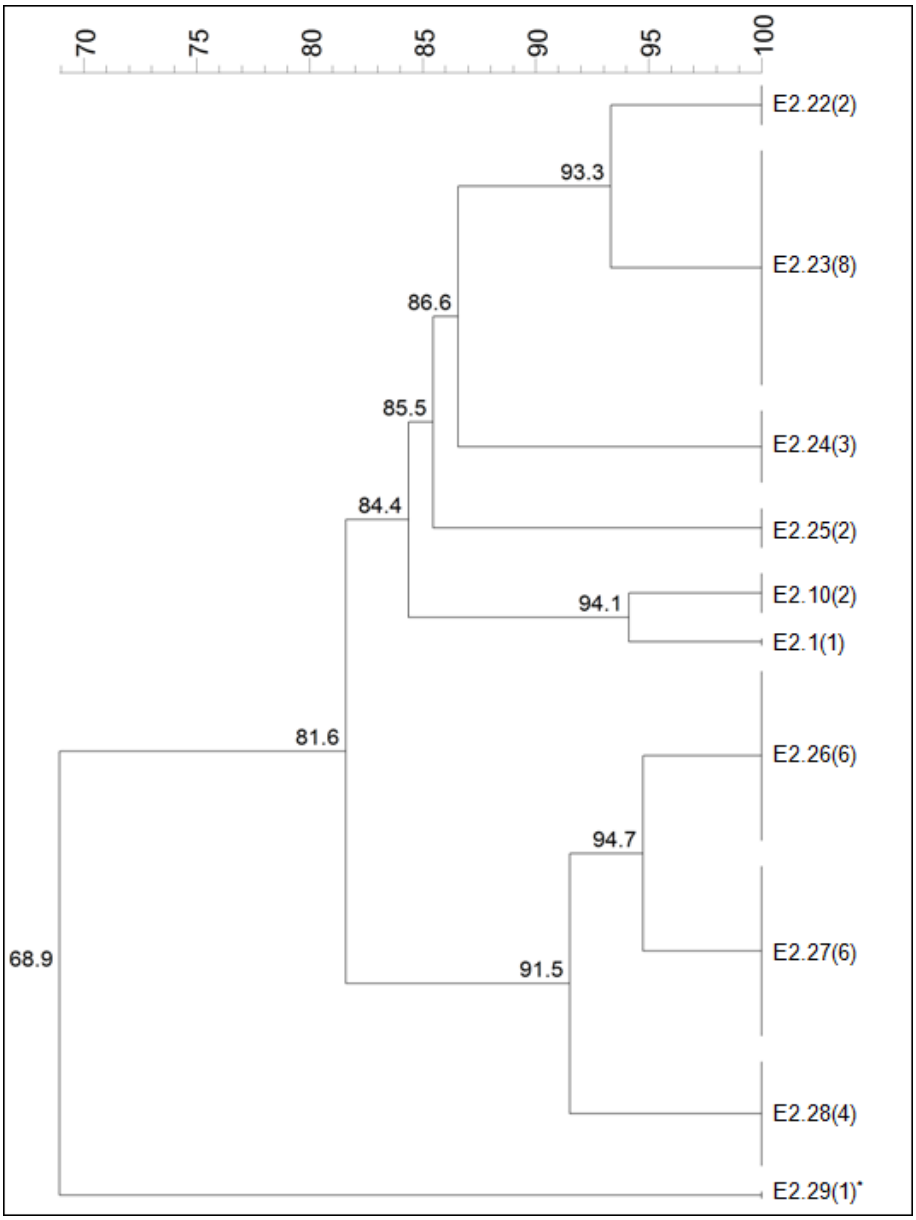

Figure 2. Cluster analysis of the ERIC-PCR fingerprint (ERIC-2) of 32 *Corynebacterium pseudotuberculosis* isolates from goats in the Sertão Region of the Pernambuco State, Brazil. The *C. pseudotuberculosis* type strain ATCC 19410^T, the 1002 vaccine strain, and a field isolate of *Rhodococcus equi* were also included. The numbers in parentheses correspond to the number of isolates within the genotype. The field isolated of *R. equi* is denoted by an asterisk.

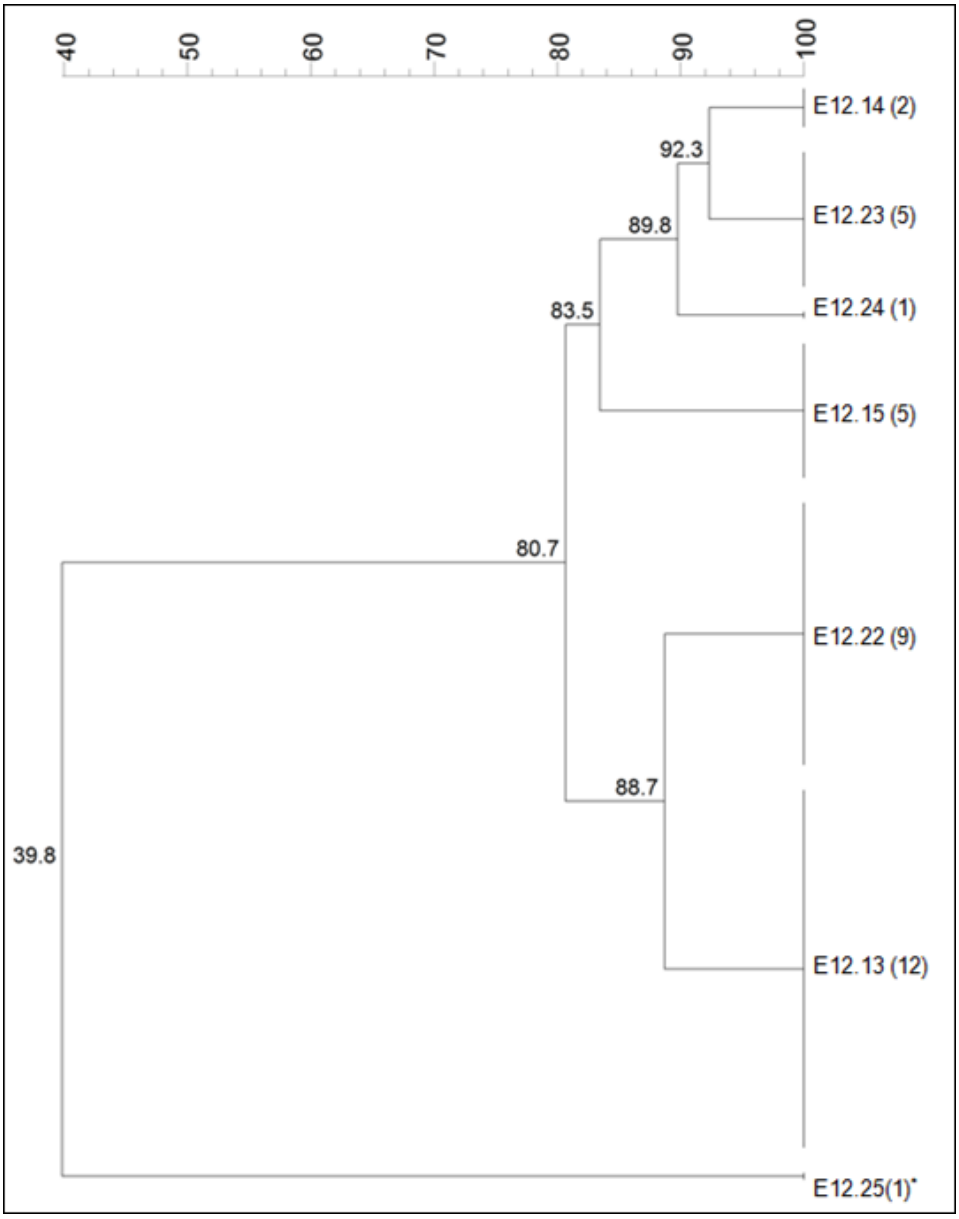

Figure 3. Cluster analysis of the ERIC-PCR fingerprint (ERIC-1+2) of 32 *Corynebacterium pseudotuberculosis* isolates from goats in the Sertão Region of the Pernambuco State, Brazil. The *C. pseudotuberculosis* type strain ATCC 19410^T, the 1002 vaccine strain, and a field isolate of *Rhodococcus equi* were also included. The numbers in parentheses correspond to the number of isolates within the genotype. The field isolated of *R. equi* is denoted by an asterisk.

were fingerprinted using ERIC-PCR and assigned a type. For each ERIC-PCR assay, the numbers of genotypes found in the municipalities of Floresta and Ibimirim were, respectively, as follows: ERIC-1-PCR, 4 and 6; ERIC-2-PCR, 4 and 8; ERIC-1+2-PCR, 2 and 6.

Of the 8 genotypes identified with ERIC-1-PCR among the *C. pseudotuberculosis* isolates from goats, 3 had been previously described in sheep in Minas Gerais State, Brazil (Guimarães et al., 2011b). The new genotypes constituted 37.14% of all typed *C. pseudotuberculosis* goat isolates. In the ERIC-2-PCR assay, only 2 genotypic profiles matched previously reported genotypes (Guimarães et al., 2011b), whereas 85.71% of the studied goat isolates had not been previously described. Fingerprinting of *C. pseudotuberculosis* isolates from goats using ERIC-1+2-PCR also revealed 3 genotypes that were identical to genotypes previously described in *C. pseudotuberculosis* isolates from sheep (Guimarães et al., 2011b), whereas 45.71% of the strains represented new genotypes. For all ERIC-PCR assays, the previously described genotypes were assigned the same identification label used by Guimarães et al. (2011b), and the new genotypes were labeled sequentially in the same way (see Figures 1, 2, and 3). The 1002 vaccine strain and the ATCC 19410^T strain showed genotypes similar to those of most isolates tested and identical to 2 described by Guimarães et al. (2011b), whereas the *R. equi* field strain exhibited a pattern that was genetically distinct from the *C. pseudotuberculosis* strains (see Figures 1, 2, and 3).

DISCUSSION

Until recently, published data have shown that the techniques (pulse-field gel electrophoresis (Connor et al., 2000), restriction fragment length polymorphism (Sutherland et al., 1996), ribotyping (Sutherland et al., 1993), and RAPD (Foley et al., 2004)) available for the molecular characterization of *C. pseudotuberculosis* strains isolated from various hosts have low discriminatory power. In contrast, ERIC-PCR has been successfully used in our research to genotype *C. pseudotuberculosis* isolates from sheep, indicating that it is a promising technique for use in CLA epidemiology (Guimarães et al., 2011b). The findings of the present study corroborate those of the previous report, demonstrating high discriminatory power and typeability for all ERIC-PCR assays tested on *C. pseudotuberculosis* isolates from goats; furthermore, our results enabled us to make epidemiological inferences.

HGDI is a key characteristic of typing systems and is based on an estimate of their capability to differentiate between 2 unrelated strains (Hunter, 1990). The high HGDIs of the 3 ERIC-PCR assays used on the *C. pseudotuberculosis* goat isolates were similar to those reported by Guimarães et al. (2011b) in sheep isolates. These results demonstrated the applicability and high discriminatory power of this typing technique compared to those previously used with *C. pseudotuberculosis*. Despite the relatively small number of strains studied (32), the ERIC-PCR assays facilitated differentiation among strains, yielding 7 to 10 distinct genotypes depending on the primer set used.

The ERIC-PCR typing data suggest that extensive genetic diversity exists among *C. pseudotuberculosis* isolates from Ibimirim and Floresta municipalities in the Sertão Region of Pernambuco State, Brazil. This genetic heterogeneity may reflect the current rapid growth of goat and sheep flocks in Brazil, which has resulted in the mass transportation of animals for breeding and the establishment of new herds. From 1997 to 2007, the goat and sheep herds in Brazil increased by 18.6 and 11.7%, respectively (Instituto Brasileiro de Geografia e Estatística (IBGE), 2007). In the same period, the sheep and goat herds in the northeast region,

which includes the State of Pernambuco, grew by 29.6 and 16.4%, respectively (IBGE, 2007). In addition, in the herds sampled, sheep and goats frequently shared the same pasture.

The genotypes observed among the goat isolates of *C. pseudotuberculosis*, which were principally revealed in the ERIC-1-PCR and ERIC-1+2-PCR assays, showed that most of the goat isolate genotypes were similar to those of the *C. pseudotuberculosis* isolates from sheep in Minas Gerais State (Guimarães et al., 2011b). This high degree of similarity among *C. pseudotuberculosis* isolates from goats and sheep in 2 states in different regions in Brazil could also be explained by the rapid growth of the sheep herd in Brazil. Minas Gerais State received most of the sheep used to establish new flocks from the northeast region, which has the largest sheep herd in the country (Guimarães et al., 2009, 2011b). Moreover, most of the farms raised goats and sheep in the same herd, which could increase the rate of exchange of *C. pseudotuberculosis* strains between goats and sheep. These results are also supported by the high prevalence of CLA found in goat and sheep flocks in the State of Minas Gerais (Guimarães et al., 2009; Seyffert et al., 2010) and in goat flocks in the State of Ceará, which is also in the northeast region (Pinheiro et al., 2000).

The novel genotypes (Guimarães et al., 2011b) observed in this study may have resulted from differences between hosts (goats *vs* sheep), locations (Pernambuco *vs* Minas Gerais State), or sampling times (2007 *vs* 2010). Future studies using a larger collection of related and unrelated *C. pseudotuberculosis* strains isolated from various hosts should be carried out to demonstrate the epidemiological usefulness of the ERIC-PCR typing method.

The *C. pseudotuberculosis* vaccine strain 1002 [Empresa Brasileira de Pesquisa Agropecuária (Embrapa), 2000] showed high genetic similarity to *C. pseudotuberculosis* strains isolated from goats and sheep; these results suggest that the 1002 vaccine may protect against infection from *C. pseudotuberculosis* of various origins. It is unlikely that any of the strains typed in this or the previous study could be field isolates of the 1002 vaccine, as vaccination levels are still very low; the vaccine was not used in any of the flocks in Minas Gerais State in 2002 (Guimarães et al., 2009) and in only about 12% in 2007.

The results of the present study showed that ERIC-PCR has good discriminatory power and typeability, making the method useful for discriminating among *C. pseudotuberculosis* isolates from goats. The results highlight the usefulness of this method for investigating epidemiological relationships and sources of infection, and the method could thus be used to improve the control of CLA.

ACKNOWLEDGMENTS

A.P. Lage, E.M.S. Dorneles, E.L.S. Santos, J.A. Santana, M.B. Heinemann, and V. Azevedo are indebted to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for fellowships received. Research supported by CNPq, Fundação de Amparo à Pesquisa do Estado de Minas Gerais, and Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais. G.I. Andrade received support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior ProDoc fellowship.

REFERENCES

Baird GJ and Fontaine MC (2007). *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. *J. Comp. Pathol.* 137: 179-210.

Connor KM, Quirie MM, Baird G and Donachie W (2000). Characterization of United Kingdom isolates of *Corynebacterium pseudotuberculosis* using pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 38: 2633-2637.

Coyle MB, Hollis DG and Groman NB (1985). *Corynebacterium* spp. and Other Coryneform Organisms. In: Manual of Clinical Microbiology. (Lennette EH, Balows A, Hausler WJ and Shadomy HJ, eds.). 4th edn. American Society for Microbiology, Washington, 198-199.

Empresa Brasileira de Pesquisa Agropecuária (Embrapa) (2000). Vacina da EBDA é Novidade Mundial. Boletim Técnico, Maio/Junho.

Foley JE, Spier SJ, Mihalyi J, Drazenovich N, et al. (2004). Molecular epidemiologic features of *Corynebacterium pseudotuberculosis* isolated from horses. *Am. J. Vet. Res.* 65: 1734-1737.

Guimarães AS, Seyffert N, Bastos BL, Portela RWD, et al. (2009). Caseous lymphadenitis in sheep flocks of the state of Minas Gerais, Brazil: prevalence and management surveys. *Small Ruminant Res.* 87: 86-91.

Guimarães AS, Carmo FB, Pauletti RB, Seyffert N, et al. (2011a). Caseous lymphadenitis: epidemiology, diagnosis, and control. *Inst. Integr. Omics Appl. Biotechnol. J.* 2: 33-43.

Guimarães AS, Dorneles EM, Andrade GI, Lage AP, et al. (2011b). Molecular characterization of *Corynebacterium pseudotuberculosis* isolates using ERIC-PCR. *Vet. Microbiol.* 153: 299-306.

Hunter PR (1990). Reproducibility and indices of discriminatory power of microbial typing methods. *J. Clin. Microbiol.* 28: 1903-1905.

Hunter PR and Gaston MA (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26: 2465-2466.

Instituto Brasileiro de Geografia e Estatística (IBGE) (2007). Diretoria de Pesquisas, Coordenação de Agropecuária, Pesquisa da Pecuária Municipal 2007. Available at [http://www.ibge.gov.br]. Accessed July, 2011.

MacFaddin JF (1980). Pruebas Bioquímicas para la Identificacion de Bactérias de Importância Clinica. Panamericana, Buenos Aires.

Pacheco LGC, Pena RR, Castro TLP, Dorella FA, et al. (2007). Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples. *J. Med. Microbiol.* 56: 480-486.

Pinheiro RR, Gouveia AMG, Alves FSF and Haddad JPA (2000). Aspectos epidemiológicos da caprinocultura cearense. *Arq. Bras. Med. Vet. Zootec.* 52: 534-543.

Pitcher DG, Saunders NA and Owen RJ (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* 8: 151-156.

Seyffert N, Guimaraes AS, Pacheco LG, Portela RW, et al. (2010). High seroprevalence of caseous lymphadenitis in Brazilian goat herds revealed by *Corynebacterium pseudotuberculosis* secreted proteins-based ELISA. *Res. Vet. Sci.* 88: 50-55.

Struelens MJ (1998). Molecular epidemiologic typing systems of bacterial pathogens: current issues and perpectives. *Memórias do Instituto Oswaldo Cruz* 93: 581-586.

Sutherland SS, Hart RA and Buller NB (1993). Ribotype analysis of *Corynebacterium pseudotuberculosis* isolates from sheep and goats. *Aust. Vet. J.* 70: 454-456.

Sutherland SS, Hart RA and Buller NB (1996). Genetic differences between nitrate-negative and nitrate-positive *C. pseudotuberculosis* strains using restriction fragment length polymorphisms. *Vet. Microbiol.* 49: 1-9.

Versalovic J, Koeuth T and Lupski JR (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 19: 6823-6831.