



Molecular characterization of *Corynebacterium pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis* by ERIC-PCR

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ABSTRACT: The aims of the present study were (i) to genotype *Corynebacterium pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis* strains using enterobacterial repetitive intergenic consensus (ERIC-PCR), and (ii) to analyze the epidemiological relationships among isolates according to biovar (*Equi* and *Ovis*), species, host, and geographical origin of the *C. pseudotuberculosis* strains. Sixty-eight *C. pseudotuberculosis*, nine *C. silvaticum*, and one *C. auriscanis*, *C. pseudotuberculosis* ATCC® 19410™ strain and the attenuated *C. pseudotuberculosis* 1002 vaccinal strain were fingerprinted by ERIC 1+2-PCR. Field strains were isolated from various hosts (cattle, buffaloes, sheep, goats, horses, dogs, and pigs) in six countries (Mexico, Portugal, Brazil, Equatorial Guinea, Egypt, and Israel). High genetic diversity was found among the studied *Corynebacterium* spp. isolates, clustering in 24 genotypes with a Hunter & Gaston diversity index (HGDI) of 0.937. The minimal spanning tree of *Corynebacterium* spp. revealed three clonal complexes, each associated with one bacterial species. Twenty-two genotypes were observed among *C. pseudotuberculosis* isolates, with an HGDI of 0.934. Three major clonal complexes were formed at the minimal spanning tree, grouped around the geographic origin of *C. pseudotuberculosis* isolates. These results reinforce the high typeability, epidemiological concordance, and discriminatory power of ERIC-PCR as a consistent genotyping method for *C. pseudotuberculosis*, which could be useful as an epidemiological tool to control caseous lymphadenitis. Moreover, our results also indicate the potential of ERIC 1+2-PCR for the genotyping of other species of *Corynebacterium* other than *C. pseudotuberculosis*.

Key words: ERIC 1+2-PCR, molecular epidemiology, caseous lymphadenitis, genotyping.

Caracterização molecular de *Corynebacterium pseudotuberculosis*, *C. silvaticum* e *C. auriscanis* pelo ERIC-PCR

RESUMO: Os objetivos do presente estudo foram (i) genotipar amostras de *Corynebacterium pseudotuberculosis*, *C. silvaticum* e *C. auriscanis* usando Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), bem como (ii) analisar as relações epidemiológicas entre os isolados de acordo com biovar (*Equi* e *Ovis*), espécie, hospedeiro e origem geográfica das amostras de *C. pseudotuberculosis*. Sessenta e oito isolados de *C. pseudotuberculosis*, nove *C. silvaticum*, um *C. auriscanis*, *C. pseudotuberculosis* ATCC® 19410™ e a amostra vacinal atenuada *C. pseudotuberculosis* 1002 foram tipificadas por ERIC 1 + 2-PCR. As amostras de campo foram isoladas de diferentes hospedeiros (bovinos, búfalos, ovinos, caprinos, equinos, cães e suínos) em seis países (México, Portugal, Brasil, Guiné Equatorial, Egito e Israel). Uma alta diversidade genética foi observada entre os isolados de *Corynebacterium* spp., agrupados em vinte e quatro genótipos com um índice de diversidade Hunter & Gaston (HGDI) de 0,937. A análise da minimal spanning tree (MST) de *Corynebacterium* spp. revelou três complexos clonais, cada um associado a uma espécie bacteriana. Vinte e dois genótipos foram observados entre isolados de *C. pseudotuberculosis*, com um HGDI de 0,934. Na análise da MST, três grandes complexos clonais foram formados, agrupando-se em torno da origem geográfica dos isolados de *C. pseudotuberculosis*. Esses resultados reforçam a alta tipabilidade, concordância epidemiológica e poder discriminatório do ERIC-PCR como método consistente de genotipagem para *C. pseudotuberculosis*, podendo ser útil como ferramenta epidemiológica no controle da linfadenite caseosa. Além disso, os resultados também indicam o grande potencial de ERIC 1 + 2-PCR para genotipagem de espécies do gênero *Corynebacterium* além de *C. pseudotuberculosis*.

Palavras-chave: ERIC 1+2-PCR, epidemiologia molecular, linfadenite caseosa, genotipagem.

INTRODUCTION

The genus *Corynebacterium* comprises more than 110 bacterial species commonly found on mucous membranes, skin, or in the environment, that can cause infection in both domestic and wild animals, and in humans (OLIVEIRA et al., 2017). Infections caused by *Corynebacterium* spp. are frequently characterized by chronic suppurative lesions, which generally occur after tissue trauma (BERNARD, 2012). Among *Corynebacterium* species, *C. pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis* are of veterinary importance (COLLINS et al., 1999; GUIMARÃES et al., 2011; HACKER et al., 2016; DANGEL et al., 2020).

C. pseudotuberculosis is the etiological agent of chronic and zoonotic diseases such as caseous lymphadenitis (CLA) in sheep and goats, ulcerative lymphangitis in horses, mastitis in cattle, and oedematous skin disease in buffaloes (BARAKAT et al., 1984; SHPIGEL et al., 1993; SELIM, 2001; GUIMARÃES et al., 2011). There are two biotypes of *C. pseudotuberculosis*, classified according to host preference and nitrate reduction ability. Nitrate-negative strains correspond to biovar Ovis, which mainly infect small ruminants, whereas biovar Equi strains are nitrate-positive and infect horses and cattle (OLIVEIRA et al., 2016; ALMEIDA et al., 2017). Caseous lymphadenitis is a globally distributed disease that is highly prevalent among sheep and goats in different countries, such as Brazil and Australia (PATON et al., 2003; GUIMARÃES et al., 2009; SEYFFERT et al., 2010; GUIMARÃES et al., 2011a). Caseous lymphadenitis is responsible for significant economic losses associated with skin and carcass condemnation; wool, meat, and milk production decrease; and death (PATON et al., 2003; GUIMARÃES et al., 2011).

Since 1997, atypical strains of *C. ulcerans* have been isolated from roe deer and wild boar in Germany, causing a disease similar to caseous lymphadenitis (DANGEL et al., 2020; MÖLLER et al., 2020). The identification of these strains by biochemical methods revealed either *C. pseudotuberculosis* or invalid profiles, whereas analyses using *16S rRNA* and *rpoB* gene sequencing and MALDI-TOF suggested *C. ulcerans* to be the closest species (DANGEL et al., 2020; MÖLLER et al., 2020). Currently, these atypical *C. ulcerans* strains have been reclassified as a novel species named *C. silvaticum* sp. nov. (DANGEL et al., 2020).

Dogs can also be infected by *C. auriscanis*, which causes otitis, pyoderma, and interdigital cysts

(BYGOTT et al., 2008; HENNEVELD, 2012). However, coryneform bacteria are a part of the normal microbiota of the skin in dogs and may or may not be associated with clinical signs (AALBÆK et al., 2010).

The differential diagnosis of *C. pseudotuberculosis* and *C. silvaticum* infections presents some challenges because both microorganisms have common hosts, display similar clinical symptoms, and are difficult to separate in the laboratory because of their high phenotypic and genotypic similarity (KHAMIS et al., 2005; VENEZIA et al., 2012; SOARES et al., 2013). Certain molecular techniques have been used for typing *C. pseudotuberculosis* strains, including ribotyping (SUTHERLAND et al., 1993), restriction fragment length polymorphism (RFLP) (SUTHERLAND et al., 1996), pulsed-field gel electrophoresis (PFGE) (CONNOR et al., 2007), random amplified polymorphic DNA (RAPD) (FOLEY et al., 2004) and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) (GUIMARÃES et al., 2011b; DORNELES et al., 2012; DORNELES et al., 2014). Of these, ERIC-PCR showed a higher power and typeability when compared to other DNA-based typing methods to discriminate *C. pseudotuberculosis* strains (DORNELES et al., 2012; DORNELES et al., 2014; HAAS et al., 2017). ERIC are intergenic repetitive units, different from most other bacterial repeats, which are present with a varying number of copies and locations in the genome of several species (VERSALOVIC et al., 1991). However, despite the proven usefulness of ERIC-PCR for *C. pseudotuberculosis* typing, this molecular technique has not been tested for *C. silvaticum* and *C. auriscanis*.

Thus, considering the importance of *Corynebacterium* species in public health, the economic losses associated with caseous lymphadenitis and other clinical presentations of the infection, and their wide dissemination, the aims of the present study were (i) to genotype *C. pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis* strains using ERIC 1+2-PCR, and (ii) to analyze the epidemiological relationships among isolates according to biovar, species, host, and country of isolation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Seventy-eight field strains of *Corynebacterium* spp., *C. pseudotuberculosis* ATCC® 19410™ and the attenuated *C. pseudotuberculosis* 1002 vaccinal strain were selected for genotyping by

ERIC 1+2-PCR (Table 1). A non-probability sampling composed of 68 *C. pseudotuberculosis*, 9 *C. silvaticum*, and 1 *C. auriscanis* were tested. They were obtained from the bacterial collection of the Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, as well as from Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Semiárido, Petrolina, PE. The isolates were grown on Brain Heart Infusion (BHI) agar (HiMedia, India) supplemented with 5% defibrinated horse blood and incubated at 37 °C for 48 h. All strains were identified by biochemical tests (COYLE & LIPSKY, 1990; GUIMARÃES et al., 2011). Species identification for *C. pseudotuberculosis* was confirmed by phospholipase D (PLD) PCR (PACHECO et al., 2007). Nitrate reduction was confirmed using nitrate broth, and nitrite reduction was tested with the addition of zinc dust (FADDIN, 2000). *C. silvaticum* and *C. auriscanis* strains were identified based on Average Nucleotide Identity (ANI) analysis using FastANI v1.3 (JAIN et al., 2018) from whole genome sequencing data (unpublished data). Information on bacterial species (*C. pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis*), biovar (Ovis and Equi), host (sheep, goat, horse, pig, cattle, buffalo, and dog), and country of isolation (Mexico, Portugal, Equatorial Guinea, Brazil, Egypt, and Israel) of the *Corynebacterium* spp. strains are summarized in table 1.

DNA extraction

Corynebacterium spp. contain a specific cell wall organization that confers high resistance against lysis (DORELLA et al., 2006). Thus,

to help the cell wall rupture, the genomic DNA of *Corynebacterium* spp. strains was extracted according to the protocol described by Pitcher et al. (1989) with some additional steps. Briefly, bacterial suspensions were suspended in lysozyme solution (50 mg/mL) in TE buffer (10 mM Tris HCl; 1 mM EDTA, pH 8.0) and immediately incubated at 65 °C for 30 min (HAAS et al., 2017). During this phase (cell lysis), the samples were vortexed every 10 min. DNA concentration and quality were determined by spectrophotometry and horizontal gel electrophoresis (SAMBROOK & RUSSELL, 2001).

ERIC 1+2-PCR

All strains were fingerprinted at the same time by ERIC 1+2-PCR using the primer pair ERIC-1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (IDT, USA) as previously described (VERSALOVIC et al., 1991; GUIMARÃES et al., 2011b; DORNELES et al., 2014). The amplified products were then subjected to a single electrophoresis run (LCH-192, Locus, São Paulo, Brazil) to avoid misinterpretation associated with interassay variability.

Data analysis

Band size estimates and genotype analysis were performed using Bionumerics 7.5 (Applied Maths, Belgium). Clustering analysis was based on the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) using the same software. The strains were analyzed together (all *Corynebacterium* spp. strains) and separately (only *C. pseudotuberculosis*). The Hunter

Table 1 - Bacterial species, host, biovar, and country of origin of the 78 *Corynebacterium* spp. isolates genotyped by ERIC 1+2-PCR.

Bacterial Species	Host	Biovar		Country					
		Ovis	Equi	Brazil	Egypt	Eq. Guinea	Israel	Mexico	Portugal
<i>Corynebacterium pseudotuberculosis</i>	Cattle	1	0	0	0	0	1	0	0
	Buffalo	0	1	0	1	0	0	0	0
	Sheep	15	0	9	0	1	0	5	0
	Goat	45	1	10	0	1	0	26	9
	Horse	0	2	0	0	0	0	2	0
	Undefined	3	0	3	0	0	0	0	0
<i>C. auriscanis</i>	Dog	n/a*	n/a	0	0	0	0	1	0
<i>C. silvaticum</i>	Pig	n/a	n/a	0	0	0	0	0	9

*Not applicable.

& Gaston Diversity Index (HGDI) was calculated (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php) to evaluate the discriminatory power of ERIC 1+2-PCR for both *Corynebacterium* spp. and the *C. pseudotuberculosis* strains (HUNTER & GASTON, 1988), considering that each genotype shared 100% of similarity.

A minimal spanning tree (MST) was constructed using the UPGMA to calculate the distance matrix, Prim's algorithm associated with the priority rule, and permutation resampling (FEIL et al., 2004; SALIPANTE & HALL, 2011).

RESULTS

Identification of *Corynebacterium* spp. isolates

All 68 *C. pseudotuberculosis* species were confirmed through phenotypic and molecular tests, whereas the 9 *C. silvaticum* and 1 *C. auriscanis* species were identified based on whole genome sequencing (unpublished data). Among the *C. pseudotuberculosis* strains, 64 were classified as biovar Ovis and 4 as biovar Equi (Table 1).

ERIC 1+2-PCR Genotypes

ERIC 1+2-PCR was able to fingerprint and assign a type to all 80 *Corynebacterium* spp. strains studied (78 field strains and 2 reference strains). In the dendrogram generated by all 80 strains (*C. pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis*), the isolates were grouped into 24 genotypes (Figure 1). The HGDI calculated for ERIC 1+2-PCR considering all *Corynebacterium* spp. strains was 0.937. The HGDI is an estimator of discriminatory power of genotyping methods through determination of the ability of a typing system to differentiate between two unrelated strains (the closer to 1, the higher the probability) (STRUELENS, 1998). Of the 24 genotypes observed, 22 corresponded to *C. pseudotuberculosis*, 1 to all *C. silvaticum* strains, and 1 genotype to *C. auriscanis*. The similarity among the genotypes of *C. auriscanis*, *C. pseudotuberculosis*, and *C. silvaticum* was up to 69.4% (Figure 1), whereas a similarity of up to 76% was observed among *C. silvaticum* and *C. pseudotuberculosis* genotypes.

The different patterns among the three *Corynebacterium* species were due to bands not shared among *C. pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis* strains (Figure 1). The number of bands observed for all 9 *C. silvaticum* strains was 13, ranging from 98 to 731 bp. The *C. auriscanis* strain exhibited a genotypic pattern in the ERIC 1+2-PCR with 15 bands, ranging from 98 to 1282 bp. The average

number of bands for the 70 *C. pseudotuberculosis* strains genotyped was 9.58, ranging from 98 to 860 bp. The *C. auriscanis* strain showed three bands that were not shared with the *C. pseudotuberculosis* and *C. silvaticum* strains, and the molecular weights of these three non-shared bands were approximately 1282, 926, and 151 bp. Two bands shown by all *C. silvaticum* strains (475 and 426 bp) were not found in the profile of any *C. pseudotuberculosis* or in any *C. auriscanis*.

Analyses of the genotypes of *C. pseudotuberculosis* strains alone did not show a clustering pattern according to the isolate's host or biovar. The HGDI calculated for ERIC 1+2-PCR considering only the *C. pseudotuberculosis* strains was 0.932. The molecular weights of the most common fragments observed among *C. pseudotuberculosis* genotypes in the assay were 105, 171, 364, and 392 bp.

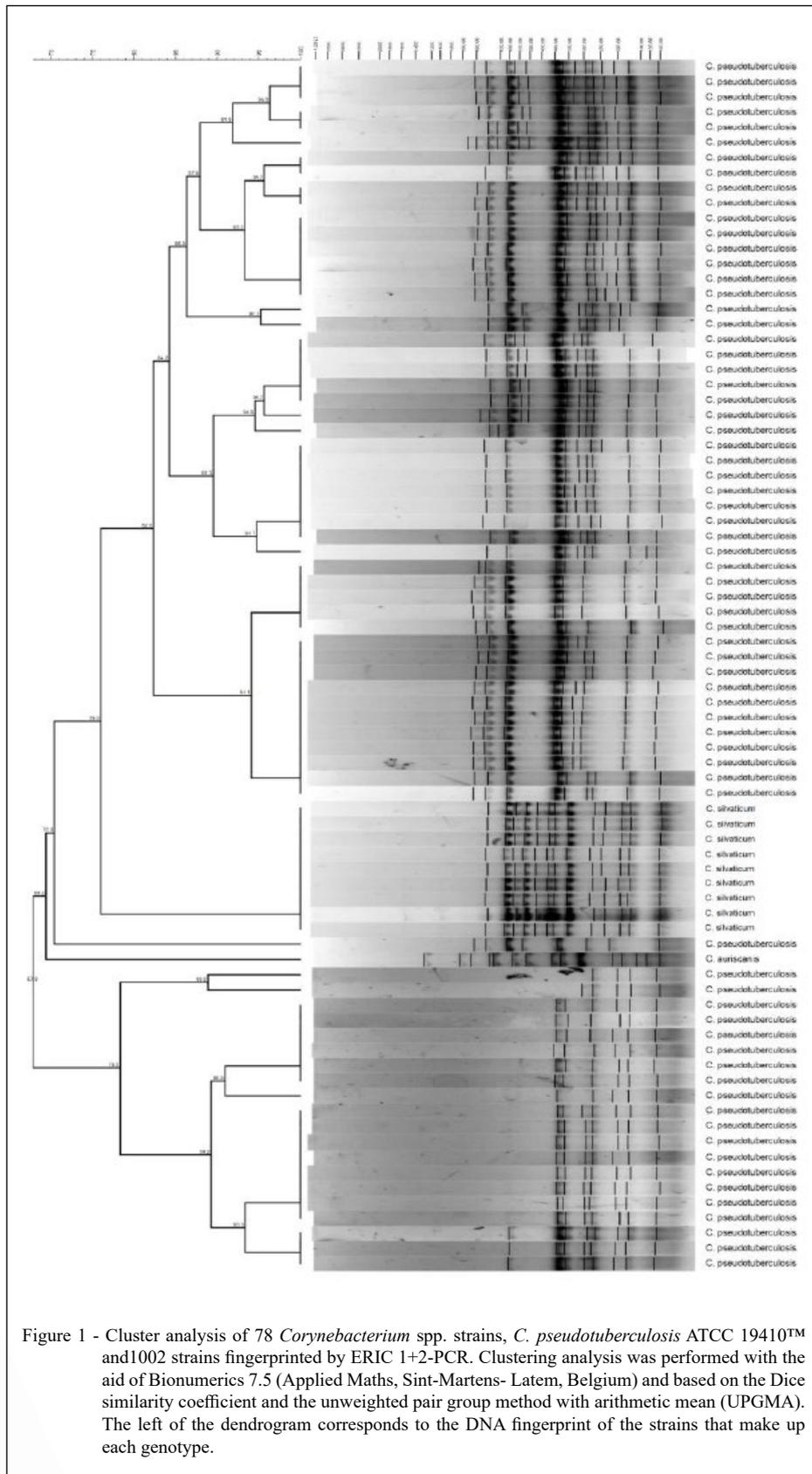
ERIC 1+2-PCR clustering patterns

A MST was constructed based on *Corynebacterium* spp. The ERIC 1+2-PCR genotypes revealed three major clonal complexes (Figure 2). In agreement with the genotypic profile exhibited on the dendrogram, the MST also displayed different clusters for the tested *Corynebacterium* species corresponding to *C. silvaticum*, *C. auriscanis*, and *C. pseudotuberculosis* strains (Figure 2). *C. pseudotuberculosis* clonal complexes were grouped into *C. silvaticum* and *C. auriscanis* clusters, with *C. silvaticum* clonal complexes located closer (5.00 length) to the *C. pseudotuberculosis* strains than the *C. auriscanis* strain was (7.00 length).

The *C. pseudotuberculosis* MST created according to the country where the strains were isolated revealed three major clonal complexes composed of strains originating from Mexico, Brazil, and Portugal (Figure 3). *C. pseudotuberculosis* strains from Israel and Egypt were grouped on the edge of the MST, while two isolates of *C. pseudotuberculosis* from Equatorial Guinea were found among the three major clonal complexes. No clustering pattern was observed for MST based on information about biovar and host of *C. pseudotuberculosis* isolates (Figures 4 and 5).

DISCUSSION

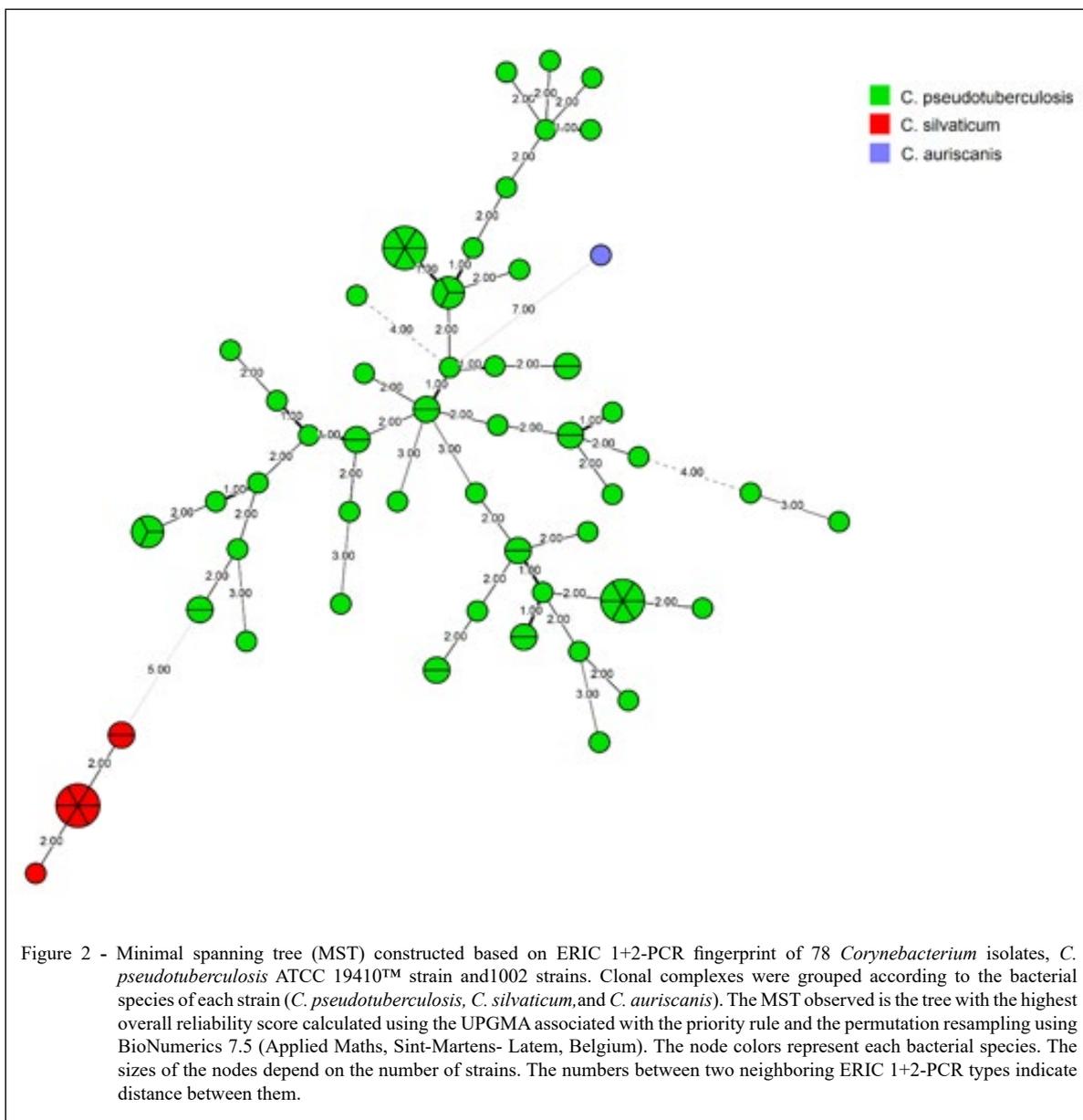
Studies have demonstrated that ERIC 1+2-PCR is an efficient technique for molecular typing of *C. pseudotuberculosis* strains isolated from different temporal data, hosts, biovars, and geographic origins (GUIMARÃES et al., 2011b; DORNELES et al., 2012; DORNELES et al., 2014; HAAS et

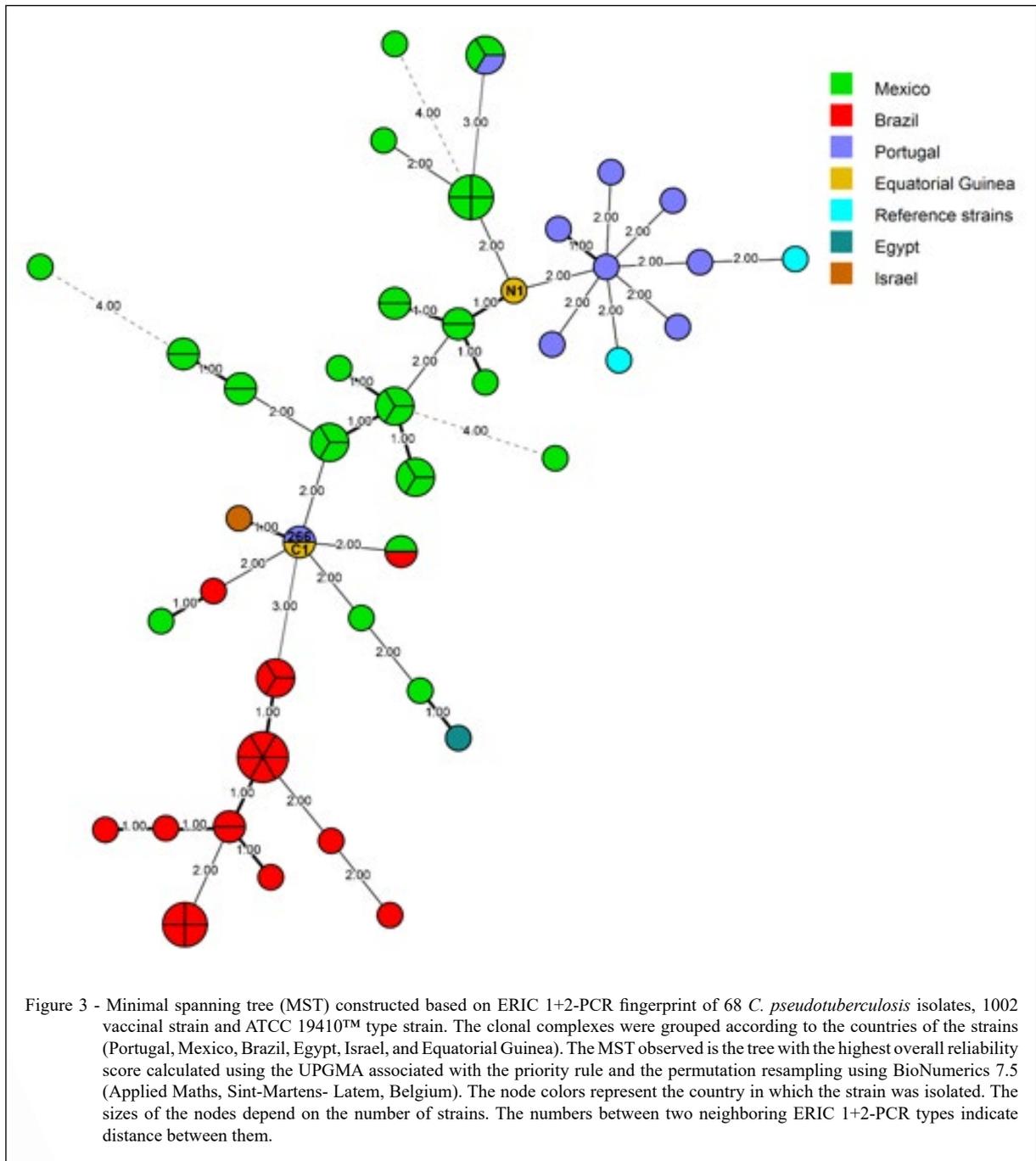


al., 2017). A good typeability was demonstrated by ERIC 1+2-PCR assignment of band patterns to all *Corynebacterium* spp. strains (*C. pseudotuberculosis*, *C. silvaticum*, and *C. auriscanis*). High typeability was observed not only for *C. pseudotuberculosis* strains, as described in previous studies, but also for other species of *Corynebacterium* that have not previously been tested by ERIC 1+2-PCR, such as *C. auriscanis* and the novel species *C. silvaticum*. Despite the small number of *C. silvaticum* and *C. auriscanis* strains

typed, these results indicate the strong potential of ERIC 1+2-PCR for genotyping *Corynebacterium* species other than *C. pseudotuberculosis*.

The previously demonstrated ERIC 1+2-PCR high genetic stability was also found in the present study, since of the four more frequent fragments observed in *C. pseudotuberculosis* strains, two were previously described by Dorneles et al. (2014) (corresponding to 392 and 105 bp) and one by Guimarães et al. (2011b) (corresponding to 171



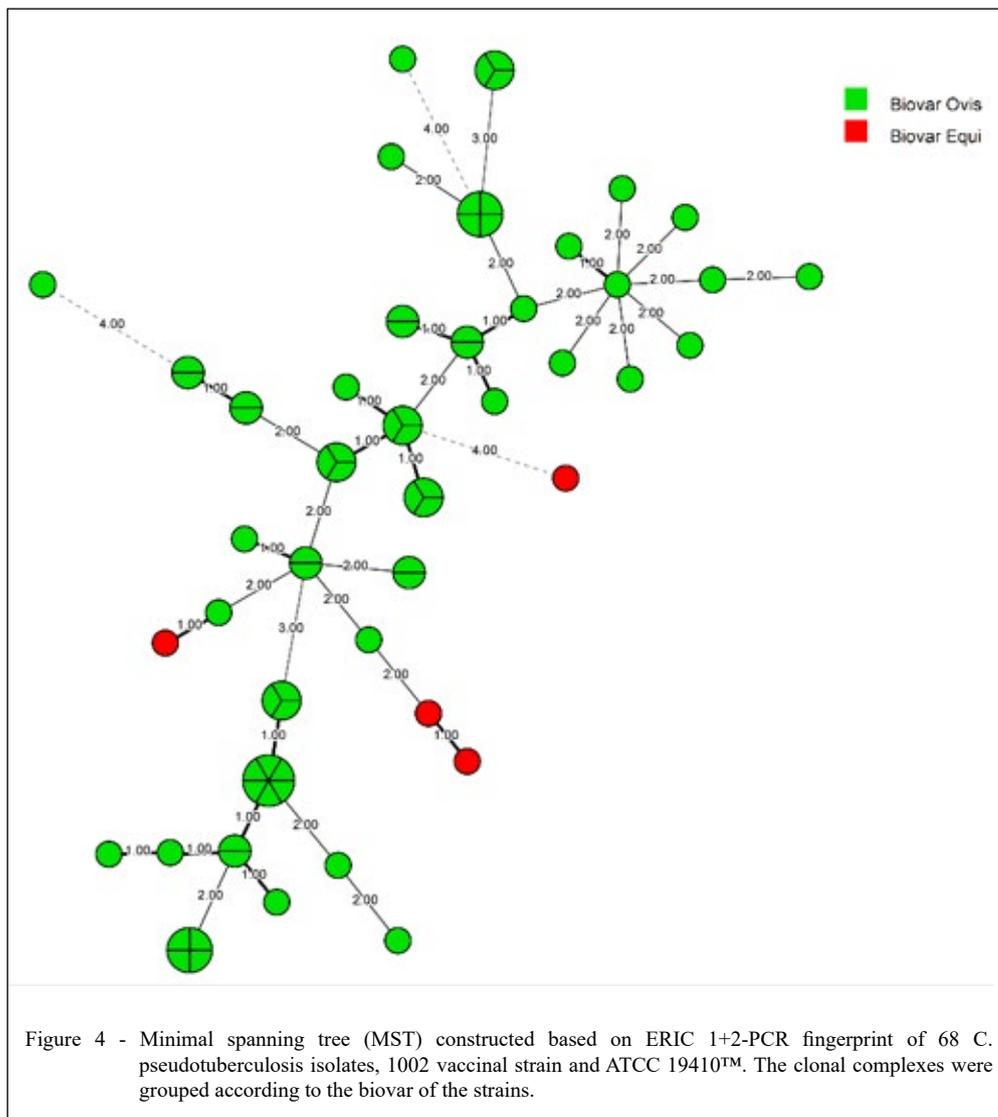


bp). The sharing of these three fragments, which were common to all strains of *C. pseudotuberculosis* analyzed in this study, demonstrates the maintenance of some band patterns over different assays even when using different strains of *C. pseudotuberculosis*, proving the strong genetic stability of the ERIC 1+2-

PCR. These results reinforce the usefulness of ERIC 1+2-PCR in evaluations of common sources of infection or transmission chains, since epidemiological concordance, the main quality of a typing technique, can only exist if there are molecular markers with some genetic stability. However, genetic stability,

although important for ensuring the establishment of some epidemiological links, may lead to a decrease in discriminatory power when present at high levels. Therefore, the high discriminatory power results of ERIC 1+2 in both analyses (*Corynebacterium* species and *C. pseudotuberculosis*), demonstrated by the absence of unrelated strains grouped by MST and by the high HGDI indexes found, provides more certainty. In fact, all three clonal complexes of *Corynebacterium* spp. observed by MST were grouped according to the three *Corynebacterium* species studied (Figure 2), while the three major clusters of *C. pseudotuberculosis* in the MST were grouped according to geographic origin (Figure 3).

Despite the high genetic diversity observed among *C. pseudotuberculosis* strains, the ERIC 1+2-PCR assay was able to discriminate the genotype pattern of this species from other *Corynebacterium* spp. strains through band patterns, which were clearly typical for each species (Figure 1). In addition to the different number of fragments among the three *Corynebacterium* species tested, another factor that allowed for the possible differentiation was the presence of specific bands for each species. *C. auriscanis* showed three fragments not shared by *C. silvaticum* or *C. pseudotuberculosis* (1282, 926, and 151 bp). Although only one isolate of this species was tested, the results were consistent and indicated



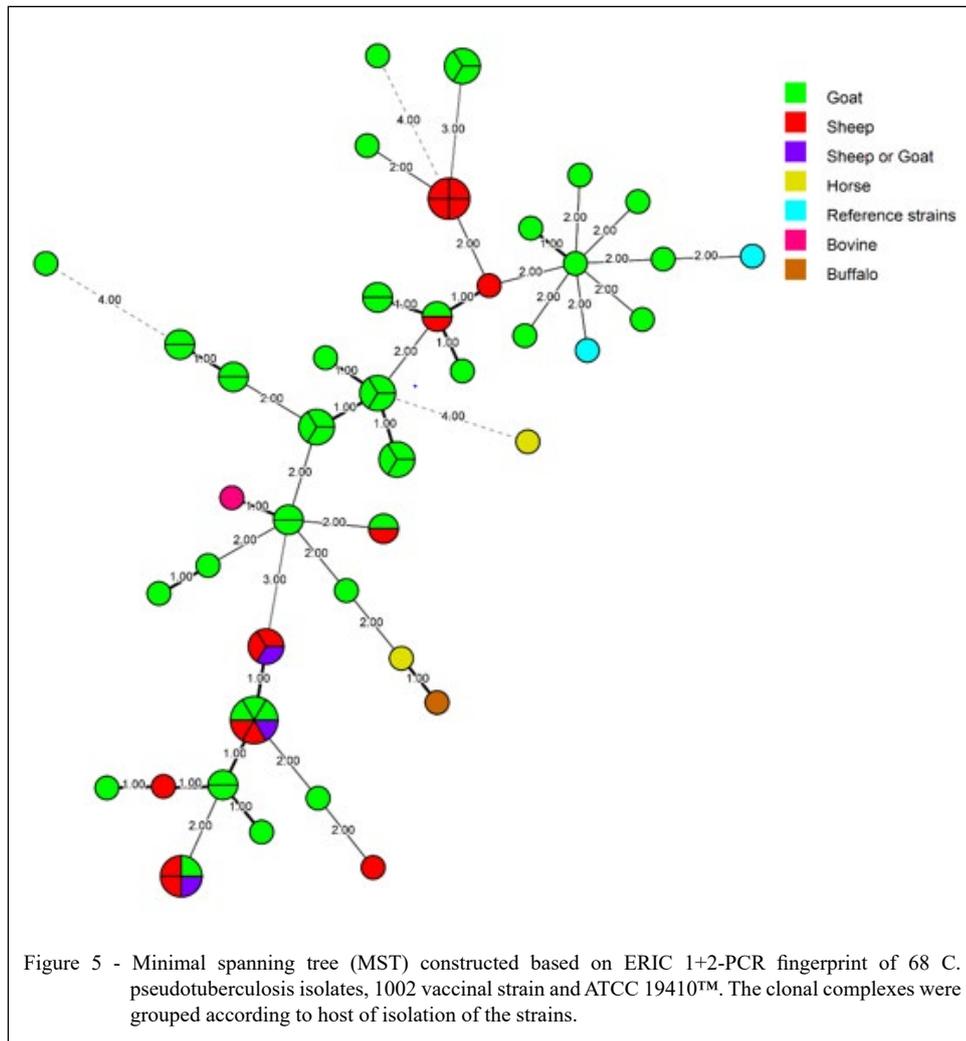


Figure 5 - Minimal spanning tree (MST) constructed based on ERIC 1+2-PCR fingerprint of 68 *C. pseudotuberculosis* isolates, 1002 vaccinal strain and ATCC 19410™. The clonal complexes were grouped according to host of isolation of the strains.

significant differentiation, which requires further investigation. Likewise, the band pattern observed for all nine *C. silvaticum* strains included two fragments not exhibited by any *C. pseudotuberculosis* strains (475 and 427 bp); therefore, the discrimination between the two species was visible and significant. These results suggest the potential of ERIC-PCR as an auxiliary tool for differentiating *C. pseudotuberculosis* from *C. silvaticum*. Challenges have been encountered in the differentiation between these species by molecular tools, such as gene-sequence-based assays, especially with 16S rRNA and *rpoB* genes, as well as MALDI-TOF typing, resulting in the proposition of this new species (DANGEL et al., 2020). To date, only whole genome sequencing data have allowed the separation of these species

(DANGEL et al., 2020). In contrast to the high genetic similarity between *C. pseudotuberculosis* and *C. silvaticum* previously observed, ERIC 1+2-PCR revealed a maximum similarity of 76 % between these two species, making their discrimination possible through a straightforward visualization. This ability to distinguish highly related species reinforces the discriminatory power and consistency of ERIC 1+2-PCR as a genotyping method for *Corynebacterium* spp. and suggests the potential use of this technique to support the differentiation between *C. pseudotuberculosis* and *C. silvaticum*.

The different genotypes for each species of *Corynebacterium* were also evidenced in the MST analysis, which revealed the existence of three clonal complexes, one for each *Corynebacterium* species

(Figure 2). The distance between the neighboring clusters in the MST indicates that *C. silvaticum* is closer to *C. pseudotuberculosis* than to *C. auriscanis*. Phylogenetic studies have not demonstrated a significant relationship between *C. auriscanis* and *C. pseudotuberculosis* by multiple-sequence alignment of the *rpoB* and 16S rRNA genes (COLLINS et al., 1999; KHAMIS et al., 2005) which corroborates the distance of 7.00 length observed between the strains of these species in the MST. The length of the lower distance of 5.00 between *C. silvaticum* and *C. pseudotuberculosis* clonal complexes was also in agreement with the greater genomic resemblance between these two species, as demonstrated in previous studies (DANGEL et al., 2020; MÖLLER et al., 2020).

The location of clonal complexes from Mexico among Brazilian and Portuguese clusters could be explained by the formation of Brazilian sheep and goat herds during colonization. Sheep and goats were introduced into America from the Iberian Peninsula by Christopher Columbus. From the Caribbean, they spread throughout the continent, and animals from Spanish-colonized countries entered Brazil (MCMANUS et al., 2010). Moreover, several breeds of goats and sheep brought to Brazil from Portugal originated local breeds, such as Canindé, Marota, Moxotó, Gurguéia goats, and Morada Nova and Santa Inês sheep, which currently comprise a considerable part of the Brazilian small ruminant herd (NOGUEIRA FILHO & KASPRZYKOWSKI, 2006), and could explain the location of strain 266 in the MST (Figure 3). There was a smaller number of isolates from Egypt, Israel, and Equatorial Guinea, and no cluster formation was observed for strains from these countries. Of the two strains from Equatorial Guinea, one was located close to Brazilian *C. pseudotuberculosis* isolates (C1), and the other next to the Portuguese strains (N1) (Figure 3). This distribution of Equatorial Guinea strains in the MST could be explained by the process of flock formation in this country, or by the characteristics of the locations of these strains. Equatorial Guinea was colonized by Portugal (1472–1778) and Spain (1778–1968); therefore, the goat and sheep flocks from Equatorial Guinea were mainly formed by animals of Portuguese and Spanish origin. The strain from Equatorial Guinea, identified as C1, was isolated from a goat of an open herd, in which animals had reported contact with goats imported from Brazil as a part of a recent attempt by the government to expand its small ruminant herd (LOUREIRO et al., 2017), which could explain its location near isolates from Brazil and Mexico. Moreover, it has the same genotype as

strain 266 isolated from a goat in Portugal, and this genotype is the link between Brazilian and Mexican *C. pseudotuberculosis* clusters. In contrast, strain N1 was isolated from a closed isolated herd, where sheep had no contact with neighboring flocks or sheep imported from Brazil (LOUREIRO et al., 2016), which explains the localization of this strain near the Portuguese *C. pseudotuberculosis* cluster in the MST, as its host would be more related to the original animals imported by the European colonizing countries.

Despite the epidemiological concordance with the geographic origin of *C. pseudotuberculosis* strains, there were no genotype patterns associated with the biovar of the strains, which contrasts with previous reports using ERIC-PCR for *C. pseudotuberculosis* (DORNELES et al., 2014). Furthermore, there was no relationship among the genotypes of the strains and the hosts. The absence of genotype patterns associated with hosts and biovars may have been related to the low representative sampling for these categories, since 4 of the 78 strains of *C. pseudotuberculosis* belonged to biovar Equi, and most isolates were from goats. Recently it has been observed that the *C. pseudotuberculosis* biovar Equi can also infect small ruminants (DOMENIS et al., 2018) and the biovar Ovis has previously been isolated from horses (RIFICI et al., 2020), showing that there is no host specificity regarding the different *C. pseudotuberculosis* biovars.

The HGDI indices observed were a consequence of the high genetic diversity of the majority of the isolates, *C. pseudotuberculosis* in particular, which showed a large number of genotypes (22 genotypes for all 68 strains). This large genetic diversity of *C. pseudotuberculosis* likely relates to the diverse geographic origin of the strains, as they were from six different countries (Mexico, Portugal, Brazil, Egypt, Israel, and Equatorial Guinea). Furthermore, the strains were isolated from five distinct hosts (goats, sheep, cattle, buffaloes, and horses). This high genetic diversity has also been observed in previous studies using ERIC-PCR for genotyping *C. pseudotuberculosis* from a heterogeneous collection of strains (DORNELES et al., 2014; HAAS et al., 2017).

The genetic diversity of the nine strains of *C. silvaticum* was very low, probably because of the isolates having the same source of infection and country, as all were isolated from pigs from Portugal. Consequently, this genetic homogeneity, supported by the shared characteristics among the strains of *C. silvaticum*, resulted in the clustering of strains into one large clonal complex of *C. silvaticum* by ERIC 1+2-PCR (Figure 2).

CONCLUSION

The occurrence of different molecular patterns according to *Corynebacterium* species and strain geographic origin, in addition to the high discriminatory power, epidemiological concordance, and typeability, proved that ERIC 1+2-PCR is a useful technique for fingerprinting *Corynebacterium* spp. strains that can be used in the investigation of outbreaks and the development of measures to prevent and control diseases, such as caseous lymphadenitis.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of this manuscript. All authors critically revised the manuscript and approved the final version.

REFERENCES

- AALBAEK, B., et al. Coryneform bacteria associated with canine otitis externa. **Vet Microbiol**, v.145, no.3-4, p.292-8. 2010. Available from: <<http://dx.doi.org/10.1016/j.vetmic.2010.03.032>>. Accessed: Nov. 23, 2020.
- ALMEIDA, S., et al. Exploration of nitrate reductase metabolic pathway in *Corynebacterium pseudotuberculosis*. **Int J Genomics**, v.2017, p.9481756. 2017. Available from: <<http://dx.doi.org/10.1155/2017/9481756>>. Accessed: Nov. 23, 2020.
- BARAKAT, A. A., et al. Two serotypes of *Corynebacterium pseudotuberculosis* isolated from different animal species. **Rev Sci Tech**, v.3, no.1, p.151-63. 1984.
- BERNARD, K. The genus *Corynebacterium* and other medically relevant coryneform-like bacteria. **J Clin Microbiol**, v.50, no.10, p.3152-8. 2012. Available from: <<http://dx.doi.org/10.1128/jcm.00796-12>>. Accessed: Nov. 23, 2020.
- BYGOTT, J. M., et al. First clinical case of *Corynebacterium auriscanis* isolated from localized dog bite infection. **J Med Microbiol**, v.57, no. Pt.7, p.899-900. 2008. Available from: <<http://dx.doi.org/10.1099/jmm.0.47780-0>>. Accessed: Nov. 23, 2020.
- COLLINS, M. D., et al. Phenotypic and phylogenetic characterization of a new *Corynebacterium* species from dogs: Description of *Corynebacterium auriscanis* sp. nov. **J Clin Microbiol**, v.37, no.11, p.3443-7. 1999.
- CONNOR, K. M., et al. Molecular genotyping of multinational ovine and caprine *Corynebacterium pseudotuberculosis* isolates using pulsed-field gel electrophoresis. **Vet Res**, v.38, no.4, p.613-23. 2007. Available from: <<http://dx.doi.org/10.1051/vetres:2007013>>. Accessed: Nov. 23, 2020.
- COYLE, M. B.; B. A. Lipsky. Coryneform bacteria in infectious diseases: Clinical and laboratory aspects. **Clin Microbiol Rev**, v.3, no.3, p.227-46. 1990.
- DANGEL, A., et al. *Corynebacterium silvaticum* sp. nov., a unique group of NTTB corynebacteria in wild boar and roe deer. **Int J Syst Evol Microbiol**, V. 70, no. 6, p. 3614-24. 2020. Available from: <<http://dx.doi.org/10.1099/ijsem.0.004195>>. Accessed: Nov. 23, 2020.
- DOMENIS, L., et al. Caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis* in Alpine chamois (*Rupicapra r. rupicapra*): A Review of 98 Cases. **J Comp Pathol**, v.161, p.11-9. 2018. Available from: <<http://dx.doi.org/10.1016/j.jcpa.2018.04.003>>. Accessed: Nov. 23, 2020.
- DORELLA, F. A., et al. *Corynebacterium pseudotuberculosis*: Microbiology, biochemical properties, pathogenesis and molecular studies of virulence. **Vet Res**, v.37, no.2, p.201-18. 2006. Available from: <<http://dx.doi.org/10.1051/vetres:2005056>>. Accessed: Nov. 23, 2020.
- DORNELES, E. M., et al. Molecular characterization of *Corynebacterium pseudotuberculosis* isolated from goats using ERIC-PCR. **Genet Mol Res**, v.11, no.3, p.2051-9. 2012. Available from: <<http://dx.doi.org/10.4238/2012.August.6.9>>. Accessed: Nov. 23, 2020.
- DORNELES, E. M., et al. Evaluation of ERIC-PCR as genotyping method for *Corynebacterium pseudotuberculosis* isolates. **PLOS ONE**, v.9, no.6, p.e98758. 2014. Available from: <<http://dx.doi.org/10.1371/journal.pone.0098758>>. Accessed: Nov. 23, 2020.
- FADDIN, J. F. M. **Biochemical Tests for Identification of Medical Bacteria**: Lippincott Williams & Wilkins. 2000.
- FEIL, E. J., et al. EBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. **J Bacteriol**, v.186, no.5, p.1518-30. 2004. Available from: <<http://dx.doi.org/10.1128/jb.186.5.1518-1530.2004>>. Accessed: Nov. 23, 2020.
- FOLEY, J. E., et al. Molecular epidemiologic features of *Corynebacterium pseudotuberculosis* isolated from horses. **Am J Vet Res**, v.65, no.12, p.1734-7. 2004. Available from: <<http://dx.doi.org/10.2460/ajvr.2004.65.1734>>. Accessed: Nov. 23, 2020.
- GUIMARÃES, A. S., et al. High sero-prevalence of caseous lymphadenitis identified in slaughterhouse samples as a consequence of deficiencies in sheep farm management in the state of Minas Gerais, Brazil. **BMC Vet Res**, v.7, p.68. 2011a. Available from: <<http://dx.doi.org/10.1186/1746-6148-7-68>>. Accessed: Nov. 23, 2020.
- GUIMARÃES, A. S., et al. Caseous lymphadenitis: Epidemiology, diagnosis, and control. **HOAB J**, v.2, no.2, p.33-43. 2011.

- GUIMARÃES, A. S., et al. Molecular characterization of *Corynebacterium pseudotuberculosis* isolates using ERIC-PCR. **Vet Microbiol**, v.153, no.3-4, p.299-306. 2011b. Available from: <<http://dx.doi.org/10.1016/j.vetmic.2011.06.002>>. Accessed: Nov. 23, 2020.
- GUIMARÃES, A. S., et al. Caseous lymphadenitis in sheep flocks of the state of Minas Gerais, Brazil: Prevalence and management surveys. **Small Rumin Res**, v.87, no.1-3, p.86-91. 2009. Available from: <<http://dx.doi.org/10.1016/j.smallrumres.2009.09.027>>. Accessed: Nov. 23, 2020.
- HAAS, D. J., et al. Molecular epidemiology of *Corynebacterium pseudotuberculosis* isolated from horses in California. **Infect Genet Evol**, v.49, p.186-94. 2017. Available from: <<http://dx.doi.org/10.1016/j.meegid.2016.12.011>>. Accessed: Nov. 23, 2020.
- HACKER, E., et al. *Corynebacterium ulcerans*, an emerging human pathogen. **Future Microbiol**, v.11, p.1191-208. 2016. Available from: <<http://dx.doi.org/10.2217/fmb-2016-0085>>. Accessed: Nov. 23, 2020.
- HENNEVELD, K. *Corynebacterium* spp. in Dogs and Cats with otitis Externa and/or Media: A Retrospective Study. **J Am Anim Hosp Assoc**, v.5, p.48, n.5, p.320-326-2012 v.2012. Available from: <dx.doi.org/10.5326/JAAHA-MS-5791>. Accessed: Nov. 23, 2020.
- HUNTER, P. R.; M. A. Gaston. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. **J Clin Microbiol**, v.26, no.11, p.2465-6. 1988.
- JAIN, C., et al. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. **Nat Commun**, v.9, no.1, p.5114. 2018. Available from: <<http://dx.doi.org/10.1038/s41467-018-07641-9>>. Accessed: Nov. 23, 2020.
- KHAMIS, A., et al. Comparison between rpoB and 16S rRNA gene sequencing for molecular identification of 168 clinical isolates of *Corynebacterium*. **J Clin Microbiol**, v.43, no.4, p.1934-6. 2005. Available from: <<http://dx.doi.org/10.1128/jcm.43.4.1934-1936.2005>>. Accessed: Nov. 23, 2020.
- LOUREIRO, D., et al. Seroprevalence of antibodies against bacterial pathogens in sheep from Equatorial Guinea. **Rev Sci Tech**, v.36, no.3, p.965-70. 2017. Available from: <<http://dx.doi.org/10.20506/rst.36.3.2728>>. Accessed: Nov. 23, 2020.
- LOUREIRO, D., et al. Complete genome sequence of *Corynebacterium pseudotuberculosis* viscerotropic Strain N1. **Genome Announc**, v.4, no.1. 2016. Available from: <<http://dx.doi.org/10.1128/genomeA.01673-15>>. Accessed: Nov. 23, 2020.
- MCMANUS, C., et al. Genetics and breeding of sheep in Brazil. **Rev Bras Zootec**, v.39, no. suppl spe, p.236-46. 2010. Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-35982010001300026&nrm=iso>. Accessed: Nov. 23, 2020.
- MÖLLER, J., et al. Phylogenomic characterisation of a novel corynebacterial species pathogenic to animals. **Antonie Leeuwenhoek**, v.113, no.8, p.1225-39. 2020. Available from: <<https://doi.org/10.1007/s10482-020-01430-5>>. Accessed: Nov. 23, 2020.
- NOGUEIRA FILHO, A.; J. W. A. Kasprzykowski. **O Agronegócio Da Caprino-Ovinocultura No Nordeste Brasileiro**: Fortaleza: BNB-ETENE. 54 p. 2006.
- OLIVEIRA, A., et al. Insight of genus *Corynebacterium*: Ascertaining the role of pathogenic and non-pathogenic species. **Front Microbiol**, v.8, p.1937. 2017. Available from: <<http://dx.doi.org/10.3389/fmicb.2017.01937>>. Accessed: Nov. 23, 2020.
- OLIVEIRA, A., et al. *Corynebacterium pseudotuberculosis* may be under anagenesis and biovar Equi forms biovar Ovis: A phylogenetic inference from sequence and structural analysis. **BMC Microbiol**, v.16, p.100-. 2016. Available from: <<http://dx.doi.org/10.1186/s12866-016-0717-4>>. Accessed: Nov. 23, 2020.
- PACHECO, L. G. C., et al. Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples. **J Med Microbiol**, v.56, no.4, p.480-6. 2007. Available from: <<http://dx.doi.org/10.1099/jmm.0.46997-0>>. Accessed: Nov. 23, 2020.
- PATON, M. W., et al. Prevalence of caseous lymphadenitis and usage of caseous lymphadenitis vaccines in sheep flocks. **Aust Vet J**, v.81, no.1-2, p.91-5. 2003. Available from: <<http://dx.doi.org/10.1111/j.1751-0813.2003.tb11443.x>>. Accessed: Nov. 23, 2020.
- PITCHER, D. G., et al. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. **Lett Appl Microbiol**, v.8, no.4, p.151-6. 1989. Available from: <<https://sfamjournals.onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.1989.tb00262.x>>. doi: 10.1111/j.1472-765X.1989.tb00262.x>. Accessed: Nov. 23, 2020.
- RIFICI, C., et al. Atypical multibacterial granulomatous myositis in a horse: First report in Italy. **Vet Sci**, v.7, no.2, p.47. 2020. Available from: <<https://www.mdpi.com/2306-7381/7/2/47>>. Accessed: Nov. 23, 2020.
- SALIPANTE, S. J.; B. G. Hall. Inadequacies of minimum spanning trees in molecular epidemiology. **J Clin Microbiol**, v.49, no.10, p.3568-75. 2011. Available from: <<http://dx.doi.org/10.1128/JCM.00919-11>>. Accessed: Nov. 23, 2020.
- SAMBROOK, J.; D. W. Russell. **Molecular Cloning: A Laboratory Manual**: Cold Spring Harbor Laboratory Press. 2001.
- SELIM, S. A. Oedematous skin disease of buffalo in Egypt. **J Vet Med B Infect Dis Vet Public Health**, v.48, no.4, p.241-58. 2001.
- SEYFFERT, N., et al. High seroprevalence of caseous lymphadenitis in Brazilian goat herds revealed by *Corynebacterium pseudotuberculosis* secreted proteins-based ELISA. **Res Vet Sci**, v.88, no.1, p.50-5. 2010. Available from: <<http://dx.doi.org/doi>>. doi: 10.1016/j.rvsc.2009.07.002>. Accessed: Nov. 23, 2020.
- SHPIGEL, N. Y., et al. An outbreak of *Corynebacterium pseudotuberculosis* infection in an Israeli dairy herd. **Vet Rec**, v.133, no.4, p.89-94. 1993.
- SOARES, S. C., et al. Genome sequence of *Corynebacterium pseudotuberculosis* biovar equi strain 258 and prediction of antigenic targets to improve biotechnological vaccine production. **J Biotechnol**, v.167, no.2, p.135-41. 2013. Available from: <<http://doi:10.1016/j.jbiotec.2012.11.003>>. Accessed: Nov. 23, 2020.
- STRUELENS, M. J. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. **Clin Microbiol Infect**, v.2, no.1, p.2-11. 1996. Available from: <<http://>>

dx.doi.org/10.1111/j.1469-0691.1996.tb00193.x>. Accessed: Nov. 23, 2020.

STRUELENS, M. J. Molecular epidemiologic typing systems of bacterial pathogens: Current issues and perspectives. **Mem Inst Oswaldo Cruz**, v.93, no.5, p.581-5. 1998.

SUTHERLAND, S. S., et al. Ribotype analysis of *Corynebacterium pseudotuberculosis* isolates from sheep and goats. **Aust Vet J**, v.70, no.12, p.454-6. 1993.

SUTHERLAND, S. S., et al. Genetic differences between nitrate-negative and nitrate-positive *C. pseudotuberculosis* strains using

restriction fragment length polymorphisms. **Vet Microbiol**, v.49, no.1-2, p.1-9. 1996.

VENEZIA, J., et al. Characterization of *Corynebacterium* species in macaques. **J Med Microbiol**, v.61, no.10, p.1401-1408. 2012. Available from: <[http://doi: 10.1099/jmm.0.045377-0](http://doi:10.1099/jmm.0.045377-0)>. Accessed: Nov. 23, 2020.

VERSALOVIC, J., et al. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. **Nucleic Acids Res**, v.19, no.24, p.6823-31. 1991. Available from: <<http://dx.doi.org/10.1093/nar/19.24.6823>>. Accessed: Nov. 23, 2020.