ISOLATION, CLONING AND PHYLOGENETIC ANALYSIS OF *pld* AND *cp40*, VIRULENCE FACTORS OF A MEXICAN ISOLATE OF *Corynebacterium pseudotuberculosis ovis* †

[AISLAMIENTO, CLONAJE Y ANÁLISIS FILOGENÉTICO DE *pld* Y *cp40*, FACTORES DE VIRULENCIA DE UN AISLADO MEXICANO DE *Corynebacterium pseudotuberculosis ovis*]

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SUMMARY

Background. Caseous lymphadenitis is a worldwide distribute disease that affects the sheep and goat industry. *Corynebacterium pseudotuberculosis ovis* is a facultative intracellular Gram-positive bacterium, considered the etiologic agent of the disease. Complete genome sequences of Mexican isolates have been obtained and different strain has been previously characterized. The study of virulence factors allows establishing potential candidates for the development of vaccines and diagnostic tools. **Objective.** To identify the complete *pld* and *cp40* genes sequence from a Mexican isolate of *Corynebacterium pseudotuberculosis ovis*, principal virulence factors. **Methodology**. *Corynebacterium pseudotube*rculosis isolate 2J-L was obtained from an abscess of a sheep of the State of Jalisco. The complete *pld* and *cp40* genes were amplified by PCR, cloned into a replicative vector and sequenced by Sanger automatic sequencing. Gene sequences conservancy was established, analyzing homology across previously reported genes of Mexican strains MEX1, MEX2, MEX9, MEX25 and MEX29. **Results**. Sequences of *pld* and *cp40* genes from isolate 2J-L presented high percentages of similarity (99%) in comparison with the sequences of other isolates of *Corynebacterium pseudotuberculosis*, reported in the GenBank database. The analysis of nucleotide sequence homology and phylogenetic tree based on *pld* and *cp40* directed the observation that 2J-L is related to Mexican strain MEX29 and MEX25. Phylogenetic results agreed on the idea that strains biovar *ovis* and biovar *equi* are groupings on different clades. Finally, results indicate that Mexican strains are more similar among strains isolated from the same host type, without geography distance influence. **Implications.** The analysis pointed out that both genes conserve their sequences in comparison with Mexican and international strains, which encourages the research continuity for vaccine and diagnostic tools development using proteins PLD and CP40 as antigen targets. **Conclusions.** The complete *pld* and *cp40* genes from Mexican isolate 2J-L were amplified, cloned and analyzed; important virulence factors from *Corynebacterium pseudotuberculosis*.

Key words: Caseous lymphadenitis; *Corynebacterium pseudotuberculosis;* Mexican isolate; *pld*; *cp40*.

RESUMEN

Antecedentes. La linfadenitis caseosa es una enfermedad ampliamente distribuida a nivel mundial que afecta a la industria del ganado ovino y caprino. *Corynebacterium pseudotuberculosis ovis* es una bacteria Gram-positiva intracelular facultativa, considerada el agente etiológico de la enfermedad. Secuencias del genoma completo de aislados mexicanos se han obtenido y caracterizado previamente. El estudio de factores de virulencia permite establecer candidatos potenciales para el desarrollo de vacunas y herramientas de diagnóstico. **Objetivo**. El objetivo de este trabajo fue identificar la secuencia completa de los genes *pld* y *cp40* de un aislado mexicano de *Corynebacterium pseudotuberculosis ovis*, principales factores de virulencia. **Metodología.** El aislado 2J-L de *Corynebacterium pseudotuberculosis* se obtuvo de un absceso proveniente de una oveja del Estado de Jalisco. Los genes *pld* y *cp40* se

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amplificaron completos mediante PCR, se clonaron en un vector replicativo y se secuenciaron mediante secuenciación automática de Sanger. Se estableció la conservación de las secuencias de los genes *pld* y *cp40*, analizando la homología en comparación con los genes de las cepas mexicanas MEX1, MEX2, MEX9, MEX25 y MEX29 reportadas previamente. **Resultados.** Las secuencias de los genes *pld* y *cp40* del aislado 2J-L presentaron altos porcentajes de similitud (99%) en comparación con las secuencias de otros aislamientos de *Corynebacterium pseudotuberculosis*, reportadas en la base de datos GenBank. El análisis de homología de secuencia de nucleótidos y árbol filogenético basado en *pld* y *cp40*, dirigió la observación de que 2J-L está relacionado con las cepas mexicanas MEX29 y MEX25. Los resultados filogenéticos coincidieron en la idea de que las cepas biovar *ovis* y biovar *equi* se agrupan en diferentes grupos filogenéticos. Finalmente, los resultados indican que las cepas mexicanas se agrupan más cerca entre cepas aisladas del mismo tipo de hospedero, sin influencia de la distancia geográfica. **Implicaciones.** El análisis apuntó que ambos genes conservan sus secuencias en comparación con cepas mexicanas e internacionales, lo que incentiva la continuidad de la investigación para el desarrollo de vacunas y herramientas de diagnóstico utilizando las proteínas PLD y CP40 como dianas antigénicas. **Conclusiones.** Los genes completos *pld* y *cp40* del aislado Mexicano 2J-L se amplificaron, se clonaron y analizaron; importantes factores de virulencia de *Corynebacterium pseudotuberculosis*.
Palabras clave: Linfadenitis Caseosa; *Corynebacterium pseudotuberculosis;* aislado Mexicano; *pld, cp40*.

INTRODUCTION

Corynebacterium pseudotuberculosis biovar *ovis* is the causal agent of Caseous Lymphadenitis (CLA), a dangerous contagious chronic disease that affects small ruminants, being the reason for great economic losses for the livestock industry (Bernard, 2012). CLA conduce to a deterioration in the organic condition of infected animals, reduction in milk and meat production (Schreuder *et al.*, 1990; Collett *et al*., 1994), skin and carcasses condemnation and reproductive disorders (Odhah *et al*., 2019; Faeza *et al*., 2019). *C. pseudotuberculosis* is considered an emergent health problem (Moussa *et al*., 2016), characterized by lesions associated with encapsulated abscesses that prevent the action of antibiotics and indiscriminate use of them have generated multi-resistant strains (Gallardo *et al*., 2019). *C. pseudotuberculosis* is a Gram-positive, facultative intracellular bacteria, belongs to the order *Actynomicetales* and family *Corynebacteriaceae* (Bernard, 2012), representing an important pathogen responsible for a large variety of diseases among humans (Trost *et al*., 2010), ovine and caprine (Parise *et al*., 2018), equine (Muñoz *et al*., 2016), bovine (Viana *et al.*, 2017), dromedary (Tejedor *et al*., 2000), alpaca (Sprake and Gold, 2012) and llama (Lopes *et al*., 2012). The conventional protocols conducted in farms for establishing the prevention and control are still inefficient (Windsor *et al.*, 2014; Punjataewakupt *et al.*, 2019). Despite the ongoing success of classical vaccines for controlling different bacterial diseases, commercial vaccines for CLA are still facing the difficulty of not developing safety and effective protection. Glanvac™ series (Zoetis, Australia), Caseous D - T™ and Case - Bac™ (Colorado Serum Company, USA), Biodectin™ (Zoetis, España), LinfoVac (Laboratory Vencofarma, Brasil) are vaccines used in farms immunization schedules, been available in almost all producer countries, but remains the prevalence of the disease (Paton, 1995; Paton *et al*., 2003; Bastos *et al.*, 2012). For that reason, studies continue to identify the correct combination of molecules that active and increase the immune system, that could be used for generating a vaccine with a protective immunogenic response. The genes *pld* and *cp40* have been identified as the main virulence factors of *C. pseudotuberculosis.* The potential of PLD (exotoxin) (Correa *et al*., 2018) and CP40 (endoglycosidase) (Shadnezhad *et al*., 2016) have been evaluated in separated experimental assays, and they are capable of induced increases IgG and cellular immune mediators like INF-ɣ and IL-12. Both proteins are secreted antigens detected in culture supernatant from *C. pseudotuberculosis*, in addition, their expression has been identified during *in vivo* and *in vitro* assays (Rebouças *et al*., 2011; Correa *et al*., 2018).

Mexico has active production of small ruminants, been demonstrated by various studies the presence of CLA in different regions of the country. Six Mexican strains with different biovar, isolated from horse, goat and sheep, were complete sequenced and studied *in silico*. Besides a putative drug target was predicted and *in silico* analysis compared 46 strains showed two genes clusters (Restriction Modification system, exclusively in biovar *ovis* and CRISPR-Cas cluster, which is only present in biovar *equi* strains) able to establish the identification between different biovar (Parise *et al*., 2018). Another study carried out the identification of 57 isolates of *C. pseudotuberculosis* by bacteriological tests and the amplification of 16S rRNA, *rpoB* and *pld* genes, as well as, genes involved in virulence and pathogenicity (Guerrero *et al*., 2018). The virulence factor *pld* and *cp40* continue to be an objective of study due to their potential for diagnostic and vaccine development. The aim of the present study was to analyze virulence factors, *pld* and *cp40* from a Mexican isolate of *Corynebacterium pseudotuberculosis ovis* compared with those reported in Mexican strains from different regions.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Corynebacterium pseudotuberculosis isolate 4-2.2LJ (C43) of biovar *ovis* previously identify was renamed in this work as 2J-L and used for genetic material extraction and genes amplification (Guerrero *et al.,* 2018). This isolate was obtained from a naturally occurring case of Caseous lymphadenitis in a sheep of Jalisco State, Mexico. *Corynebacterium pseudotuberculosis* 2J-L was previously identified by biochemical miniaturized API Coryne test (Biomeriux 77 Lab., REF, 20900. France) and Multiplex PCR. In addition, the DNA of *Corynebacterium pseudotuberculosis* ATCC® 43926™ was used as a positive control for the PCR amplification of *pld* and *cp40*. Bacteria was growing in 5% sheep blood agar and incubated at 37°C for 24-48 hours in microaerophilic conditions. Culture morphology was evaluated, and Gram staining was used to confirm the purity of the colonies. A single colony was cultured in 5mL of brain heart infusion (BHI) medium (Oxoid, Hampshire, England) with microaerophilic conditions at 37 °C for 24 hours, for using the culture in DNA extraction. *Escherichia coli* strain DH5α (Invitrogen, USA) was used as a host for transformation with pGEM-T Easy vector containing *pld* and *cp40* genes. Transformed *E. coli* strains were cultivated in Luria broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) containing 100μg of ampicillin per mL.

Genomic DNA extraction from *C. pseudotuberculosis* 2J-L and ATCC 43926

Bacterial DNA was extracted from *C. pseudotuberculosis* 2J-L and ATCC 43926 strain using commercial Fast ID Genomic DNA Extraction Kit (Genetic ID NA, Inc. v. 8.3, USA). Accordantly with the manufacture instruction in a labeled 2mL vial was added 200mg of bacteria pellet homogenized with 1,000μL Genomic Lyse buffer premixed with Proteinase K (20mg/mL), vortex thoroughly until a homogeneous slurry was obtained. Samples were incubated at 65 °C for 30 min and centrifuged at 12,000 g for 5 min in a microcentrifuge (Corning, USA). Later 500μL of supernatant was transferred into a new labeled 2mL vial, and mixed with equal amount of Genomic Bind buffer, centrifuged at 12,000 g for 5 min. The supernatant was put through the DNA Binding Column by centrifugal force at 12,000 g for 3 min. One step of wash was performed with 800μL of Genomic Wash buffer and three steps with 800μL of 75% ethanol using centrifugation 12,000 g for 3 min. For DNA collection 100μL of elution solution was added into the column, spin at 12,000 rpm for 1 min, been DNA collected in a new 1.5 mL vial and stored at - 20^0C.

Identification of *Corynebacterium pseudotuberculosis* isolate 2J-L by PCR

Molecular identification of *C. pseudotuberculosis* was based on Multiplex PCR amplification of a partial sequence of *16s rRNA*, *rpoB*, and *pld* genes (Table 1) according to the protocol previously published (Pacheco *et al.,* 2007). Multiplex PCR was performer in a final reaction volume of 25μL containing 12.5 μL 2X Quiagen Master Mix, 1μL of each primer (100 pmol/μL) and 2μL of DNA with a concentration approximately of 30 ng/ μL. Reactions were carried out in a thermal cycler (Techne TC 512, USA) under the following conditions: initial denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 1 min, 58 °C for 40 s and 68 °C for 1 min 30 s; final extension at 68 °C for 7 min. Amplified products were resolved by electrophoresis in 1.0% (w/v) agarose, stained with ethidium bromide (0.5 mg/mL) and visualized in a transilluminator (Mini Bis Pro, DNR Bio-imagen system, USA).

Amplification of complete *pld* and *cp40* genes

To develop a primer pair specific for a complete *pld* and *cp40* genes amplification, software's Primer 3 Plus (https://primer3plus.com/) and Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) were used for oligonucleotide design based on *pld* (L16587.1) and *cp40* (JF299259) sequence from GenBank. Restriction sites were included in primers for *NcoI* (5' end) and *XhoI* (3' end) recognition, to facilitate future cloning steps (Table 2). All PCR reactions were performed in the same thermal cycler (Techne TC 512, USA) in a total reaction volume of 25μl, containing 12.5 μL 2X Quiagen Master Mix, 1μL of each primer (100 pmol/μL) and 2μL of DNA (30 ng/ μL). Amplification programs began with a denaturation step of 94°C for 5 min and ended with a final extension of 72°C for 7 min. In addition, 30 cycles for *pld* gen amplification were performed, each one involving denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and synthesis at 72°C for 2 min. For *cp40* gene amplification 35 cycles were performed under the following conditions: denaturation steps at 94°C for 1 min, annealing at 66°C for 1 min and synthesis at 72 °C for 1 min 30 sec. The amplified products were detected by ethidium bromide (0.5 mg/mL) staining after electrophoresis in 1.0% agarose gels, visualized in a transilluminator (Mini Bis Pro, DNR Bio-imagen system, USA.

Table 1. Primers targeting *16S rRNA, rpoB* and *pld* genes from *C. pseudotuberculosis*.

Gene	Primer	Sequence	pb	Final Product
16s rRNA	16s-F	ACCGCACTTTAGTGTGTGTG	20	816pb
	16s-R	TCTCTACGCCGATCTTGTAT	20	
rpoB	rpoB-F	CGTATGAACATCGGCCAGGT	20	446pb
	rpoB-R	TCCATTTCGCCGAAGCGCTG	20	
pld	Pld-F	ATAAGCGTAAGCAGGGAGCA	20	203pb
	Pld-R	ATCAGCGGTGATTGTCTTCCAGG	23	

Table 2. Primers design for *pld* and *cp40* genes amplification.

GenBank	Primers	Sequence	Restriction Enzyme	TM	pb	Final product
CP40: JF299259	CP40-F	CCATGGGTCCAACTCGCCTCGGC	*CCATGG NcoI*	60.8	23	1479pb
	CP40-R	CTCGAGCGTGCGAGTGGTGGGC	*CTCGAG XhoI*	59	22	
PLD: L16587.1	Pld-F	CCATGGCTTTTGTTGCTCTATTTATC GAA	*CCATGG NcoI*	55.7	29	1074pb
	Pld-R	CTCGAGCTCGTAGTCATTCCAACCG	*CTCGAG XhoI*	54.2	25	

Cloning strategy and sequencing

PCR products were purified using a Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) and cloned directly into pGEM-T Easy, which is supplied cut with T overhangs. According to the manufacturer's instructions plasmid and the purified PCR products were ligated with T4 DNA ligase (Promega, USA) reaction and ligation mixture was transformed into the competent cell, *Escherichia coli* DH5α (Invitrogen, USA) for plasmid propagation. Recombinants plasmid were identified by restriction enzymatic digestion with *NotI*/ *NcoI* / *XhoI*. DNA sequencing was performed by Sanger methods, on CINVESTA Center (Mexico).

Analysis of sequence data

Molecular evolutionary genetics analysis software (MEGA v10.0) was used to correct the sequences and manually placed at the same position for generating the phylogenetic trees based on *pld* and *cp40,* in comparison with sequences of *C. pseudotuberculosis* strains deposited in the database GenBank (Table 3). In addition, the analysis of the similarity and identity of the genes was performed using BLASTn. Finally, phylogenetic analyses of the 2J-L isolate were inferred using the Maximum Likelihood method and JTT matrix-based model. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with a superior log-likelihood value.

RESULTS

Identification of *Corynebacterium pseudotuberculosis* isolate 2J-L, *pld* and *cp40* genes amplification by PCR.

The identity of isolate 2J-L was confirmed as *Corynebacterium pseudotuberculosis* by Multiplex PCR (Figure 1). The complete *pld* and *cp40* genes were amplified from *Corynebacterium pseudotuberculosis* Mexican isolate 2J-L by PCR (Figure 2).

Resultant PCR fragments were ligated into the pGEM-T Easy vector and recombinant plasmids were identified by enzymatic digestion (Figure 3).

Nucleotide sequence analysis of *pld* and *cp40* from isolate 2J-L

Sequencing recombinant plasmids allowed the analysis of the sequence of *pld* and *cp40* genes from *Corynebacterium pseudotuberculosis* isolate 2J-L. Nucleotide sequence alignments allowed the identification of *pld* with 99.91% of similarities in comparison to sequences of Mexican strains MEX1(CP017711.1), MEX2(CP046644.1), MEX9 (CP014543.1), MEX25(CP013697.1) and MEX29 (CP016826.1) with a change in position 606 with a Cytosine instead of Thymine (C / T). In addition, the *cp40* gene resulted identical with a 100% of similarity to sequence of the strain MEX29, and 99.91% in

comparison with MEX1, MEX2, MEX9 with a variation in position 60 (C / T) and a variation in position 739 (G / C) respect to MEX25 (Figure 4). Sequences obtained in this study have been deposited in the GenBank database under the accession number *pld 2JL*: OL347711 and *cp40 2jL*: OL347712.

Table 3. Strains used in phylogenetic analyses were obtained from the database GenBank.

Accession No.	Strains	Biovar	Host	Clinical description	Country	Reference
CP002097.2	FRC 41	Ovis	Human	Necrotizing lymphadenitis	France	Trost *et al*., 2010
KY041980.1	Endo CP40 gene	Ovis	Sheep	Lymphoid tissue	Germany	Shadnezhad *et al*., 2016
CP017711.1	MEX1	Ovis	Goat	Retropharyngeal abscess	Mexico	Parise *et al*., 2018
CP014543.1	MEX9	Ovis	Goat	Prescapular abscess	Mexico	Parise *et al*., 2018
CP013697.1	MEX25	Ovis	Sheep	Parathyroid abscess	Mexico	Parise *et al*., 2018
CP016826.1	MEX29	Ovis	Sheep	Retropharyngeal abscess	Mexico	Parise *et al*., 2018
CP020356.1	Sigma E	Ovis	Goat	Cheesy abscess	Brazil	Pacheco *et al*., 2012
NZ_CP035678	OVIZ01	Ovis	Sheep	Lymph node	Brazil	Aquino de Sá *et al*., 2013
CP015100.2	T1	Ovis	Goat	Granulomatous lesión	Brazil	Almeida *et al*., 2016
CP019769.1	MIC6	Ovis	Sheep	Abscess	Brazil	Direct Sub/Sousa *et al*., 2017
NC_017300	1002	Ovis	Goat	LCA abscess	Brazil	Ruiz *et al.*, 2011
CP010795.2	29156	Ovis	Bovine	Cutaneous lesión	Israel	Sousa *et al.*, 2019a
NZ_LR590479.1	NCTC4681	Ovis	Sheep	-	Australia	Direct Sub/2019
CP046644.1	MEX2	Ovis	Goat	-	Mexico	Direct Sub/Sousa *et al*., 2019b
NZ_CP017291.1	MEX30	Equi	Horse	Pectoral muscle	Mexico	Muñoz *et al*., 2016
NZ_CP017292.1	MEX31	Equi	Horse	Pectoral muscle	Mexico	Muñoz *et al*., 2016
CP012022.2	262	Equi	Bovine	Ulcerative lymphangitis	Belgium	Araújo *et al*., 2016
CP003652.3	Cp162	Equi	Camel	Neck abscess	United Kindong	Hassan *et al*., 2012
CP003421.4	Cp31	Equi	Buffalo	Abscess	Egypt	Silva *et al*., 2012
AB304286.1	*C. ulcerans*, 51799, pld gene	-	Human	-	Japan	Seto *et al*., 2008
AF296340.1	EndoS gene	-	Human		USA	Collin *et al*., 2001
AY376354.1	EndoE gene	-	Human	-	USA	Collin *et al*., 2001
L06331.1	EndoF2 gene	-	-	-	-	Tarentino *et al.*, 1992

Direct Sub: Direct submission on the NCBI page.

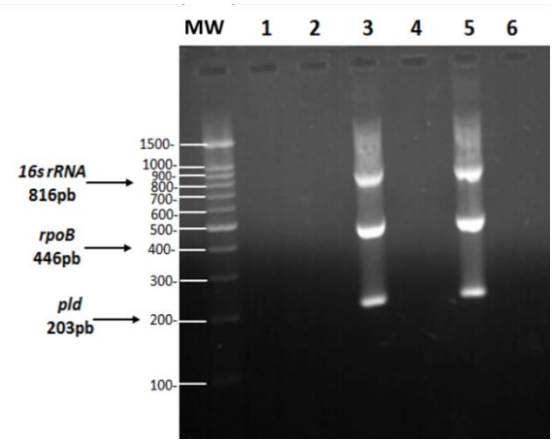

Figure 1. Identification of field isolate 2J-L of *C. pseudotuberculosis* by Multiplex PCR. MW: Molecular weight ladder, 100pb Plus DNA Ladder™ (Invitrogen, USA). Lanes 1-2: negative controls. Lanes 3-5: Amplification of partial segments of *16s rRNA, rpoB* and *pld* genes from *C. pseudotuberculosis* Lane 3: strain ATCC® 43926™ and Lane 5: isolate 2J-L.

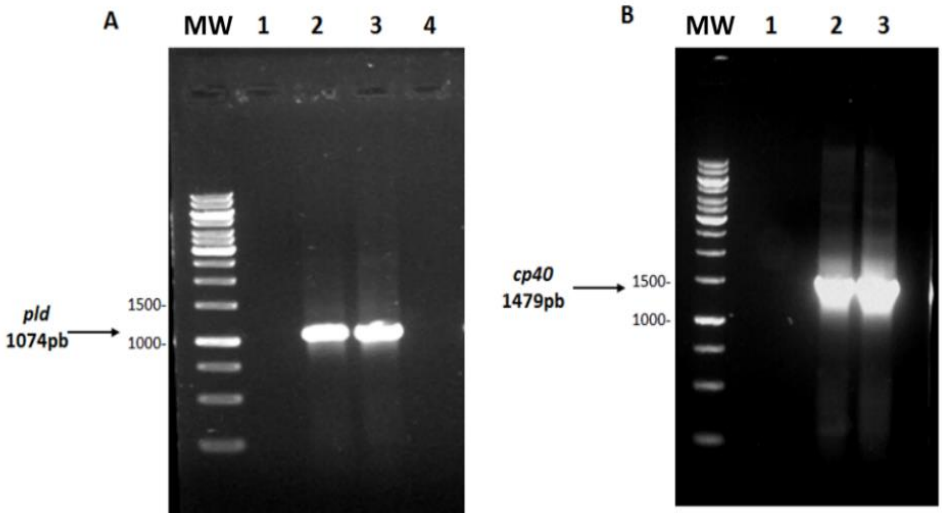

Figure 2. Amplification of *pld* (A) and *cp40* (B) genes from *Corynebacterium pseudotuberculosis* isolate 2J-L. MW: Molecular weight ladder, 1Kb O´GeneRuler (Fermentas, USA). Lane 1: negative controls, Lane 2: genes from strain ATCC® 43926™, Lane 3: genes from isolate 2J-L.

Phylogenetic analysis of *Corynebacterium pseudotuberculosis* isolate 2J-L

Phylogenetic relations of the 2J-L isolate were established based on *pld* and *cp40* sequences in comparison to other strains of *C. pseudotuberculosis,* with the construction of two separate phylogenetic trees (Figure 5). Comparative phylogenetic analysis grouped isolate 2J-L in the same clusters with biovar *ovis* strain supported by high bootstrap values, 92% for *cp40* and 99% for *pld* sequence. Both phylogenetic analyses revealed that isolate 2J-L is considerably closer to MEX29 and MEX 25 Mexican strains, included in a subgroup with 4681 and 29156 strains.

DISCUSSION

Mexico is one of the main consuming and producing countries of sheep and goat meat in America (Morales, 2020). Earlier studies have identified and sequenced the complete genome of *Corynebacterium pseudotuberculosis* strains from both biovar *ovis* and

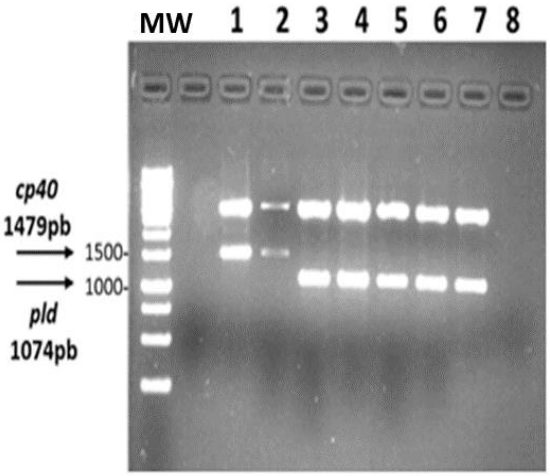

Figure 3. Enzymatic digestion assay with *NcoI/XhoI* for identification of recombinant plasmids containing the *pld* and *cp40* genes encoding the PLD and CP40 proteins of *Corynebacterium pseudotuberculosis* 2J-L. Electrophoresis on a 0.8% agarose gel. MW: Molecular weight ladder, 1Kb O´GeneRuler (Fermentas, USA). Lanes 1-2: Resultant digestion fragment of 1479 pb corresponding to plasmids modified with *cp40* gene. Lanes 3-8: Resultant fragment of 1074 pb corresponding to plasmids modified with *pld* gene.

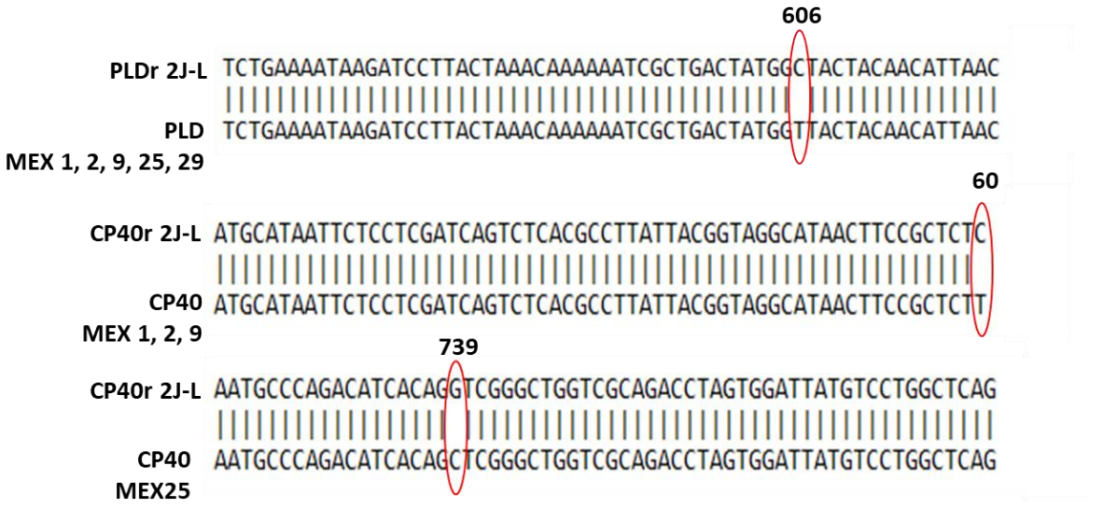

Figure 4. Alignment of sequences of *pld* and *cp40* genes of isolate 2J-L in comparison with the sequences of the Mexican strains reported in the database GenBank using the BLAST program. Lines indicate identical nucleotides and red circles represent changes in sequence. The *pld* 2J-L gene presents one change with respect to the sequence of MEX1, MEX2, MEX9, MEX25 and MEX29 strains at position 606 (C / T). The *cp40* 2J-L gene shows a change with respect to the strains MEX1, MEX2, MEX9 at position 60 (C / T) and a variation on MEX25 at position 739 (G / C).

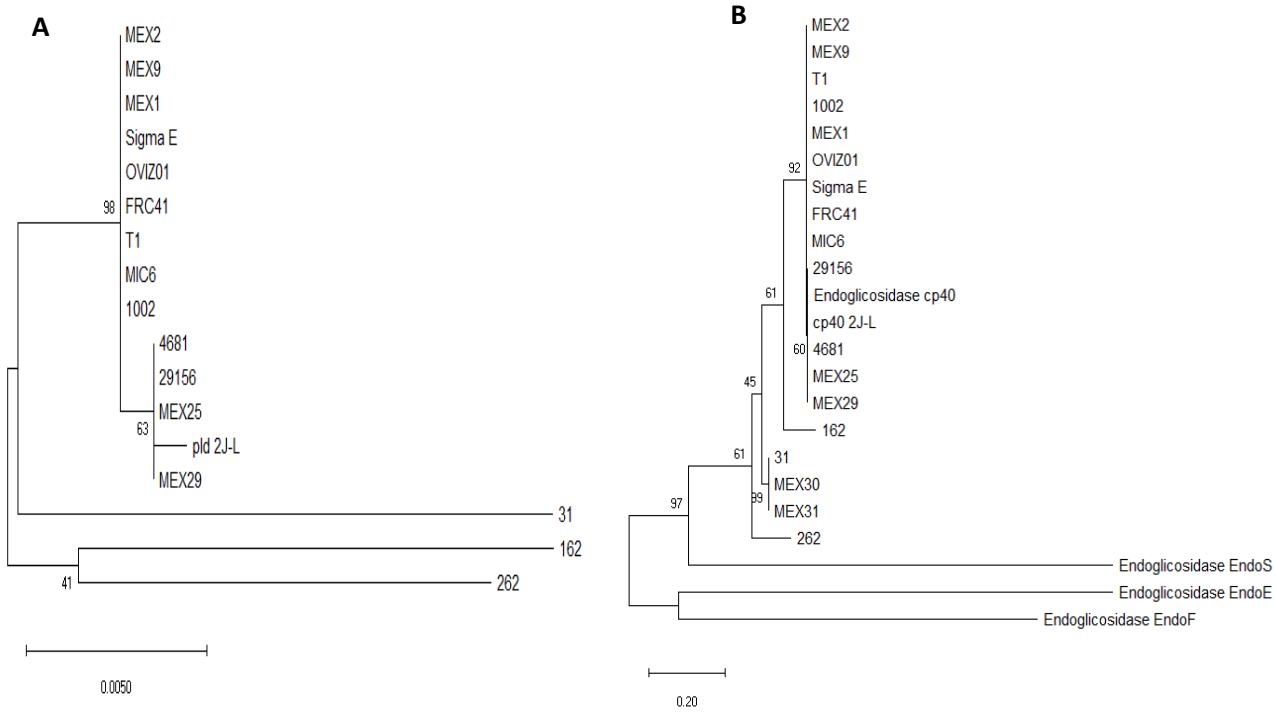

Figure 5. Phylogenetic dendrograms were constructed based on the *pld* (A) and *cp40* (B) genes of *Corynebacterium pseudotuberculosis* 2J-L in comparison to other related sequences. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with a superior log-likelihood value. Evolutionary analyses were conducted in MEGA X.

equi, obtained from outbreaks in the different States of Mexico (Parise *et al*., 2018; Muñoz *et al.,* 2016). Molecular identification, biochemical characterization and pathogenicity factors such as *fag A, fag B, fag C, fag D* and *hsp60* have been analyzed of *Corynebacterium pseudotuberculosis* isolates from Jalisco State (Guerrero *et al*., 2018). One of the samples derived from that study, sample 4-2.2LJ (C43) from sheep origin, was used in this work and renamed as isolate 2J-L, for *pld* and *cp40* identification for future use as potential candidates for vaccine and diagnostic development. Multiplex PCR products confirmed 2J-L previous identification as *C. pseudotuberculosis*, based on the amplification of partial *rpoB, 16S rRNA* and *pld.* Multiplex PCR has been validated by different authors, due to the combination of that variety of genes that allow *Corynebacterium pseudotuberculosis* differentiation concerning the closest species *Corynebacterium diphtheria* and *Corynebacterium ulceran* (Pacheco *et al.,* 2012). New primers design, PCR schedules and cloning steps used in this work were effective to obtain the complete genes and their insertion into pGEM-T Easy plasmid. These results constitute an advantage and the starting point for obtaining the proteins by recombinant technology in future assays.

Analysis of nucleotide sequence of *pld* and *cp40* genes from isolate 2J-L allowed establishing differences and similarities respect other Mexican strains previously reported. Sequences of *pld* and *cp40* genes from isolate 2J-L presented high percentages of similarity in comparison with the sequences of other isolates of *Corynebacterium pseudotuberculosis*, reported in the GenBank database. The highest percentages of identity were presented concerning Mexican strain MEX25 and MEX29, as well as with the strains 4681 from an Australian sheep and the isolate 29156 from a bovine of Israel (Sousa, 2019a). The results show that these genes are highly conserved due to the role of proteins PLD and CP40, as virulence and pathogenicity factors of *Corynebacterium pseudotuberculosis* (Hodgson *et al*., 1990; Shadnezhad *et al*., 2016).

Phylogenetic results are in concordance with those obtained by other authors where strains biovar *ovis* and biovar *equi* are grouped on different clades (Soares *et al*., 2013; Oliveira *et al*., 2016). Isolate 2J-L was grouped to Mexican strains biovar *ovis*, with preference to the type of host. Results obtained according to the analyzes of the complete genome of Mexican strains MEX1, MEX9, MEX25, MEX29, showed that strains were grouped predominantly depending on the type of host, and not due to geographical proximity. But like in other studies, also isolate 2J-L was found in the same clade with strains biovar *ovis* from different types of hosts, related in the same clade with strain 29156, isolated from a bovine of Israel (Sousa, 2019a). Isolate 2J-L was obtained from a sheep belonging to a farm with ovine and caprine coexisting together, where this farm could have different sources of biovar *ovis* strains. Biovar *ovis* strains contain a high degree of clonality, with more sequences conserved and the capacity to infect different hosts. While biovar *ovis* strains formed mix groups with a representation of different types of hosts, strains belonging to biovar *equi* are under a constant mutational process, presenting established strain differences that make their phylogenetic tree distribution more specific and separate by group, even by type of host (Soares *et al*., 2013).

Previous studies revealed that MEX1 strain (NZ_CP017711.1) was isolated from the abscess of a goat in the Tlaxcala region, MEX2 (CP046644.1) from a goat of Puebla and MEX29 strain (NZ_CP016826.1) from a sheep of the Rio Frio de Juarez, all regions with 40-50 Km approximately between isolates. In addition, strain MEX25 (NZ_CP013697.1) was isolated from a sheep and MEX9 (NZ_CP014543.1) from a goat, both isolated from the Guanajuato region, with 450 km concerning previous isolates. Isolate 2J-L was obtained from Jalisco, Guadalajara with 276 Km respect Guanajuato and 666 Km of Tlaxcala. Despite the difference in the geographic location of isolates MEX25, MEX29, and 2J-L, all derived from sheep, there were grouped in the same clade. The same behavior was observed for MEX1, MEX2 and MEX9, all derived from goats, grouped closer phylogenetically (Parise *et al*., 2018). The gene sequence analysis (*pld* and *cp40*) indicates that Mexican strains are more similar among strains isolated from the same type of host, corroborated in previous reports with the complete genome of Mexican strains (Parise *et al*., 2018).

CONCLUSIONS

Caseous Lymphadenitis remains an important disease with a tremendous negative impact on the economy of small ruminate producers in Mexico and all over the world. We provide gene sequence of *pld* and *cp40* from a *Corynebacterium pseudotuberculosis* Mexican isolate, focusing on their conserved sequence and homology compared to genes from Mexican strains previously reported. Both genes from isolate 2J-L present a conserved sequence concerning genes on Mexican strains and might be relevant for vaccine and diagnostic development to improve controls protocols for CLA in Mexico.

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Compliance with ethical standards. The authors declare that all the procedures that contributed to the realization of this work comply with the ethical standards of the UAEM, and with the authorization by a bioethical committee of the Faculty of Veterinary Medicine and Zootechnics, UAEM.

Data availability. Authors declare that all data are available with Ph.D. Roberto Montes de Oca Jiménez, romojimenez@yahoo.com

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