Genomic analysis of Mexican strains of *Anaplasma marginale* and *Anaplasma centrale*: an application for the development of a species-specific molecular detection test

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Resumen
La detección de patógenos es fundamental para la vigilancia de las enfermedades que podrían desarrollarse en el ganado bovino de nuestro país. La anaplasmosis bovina puede ser causada por *Anaplasma marginale* y *Anaplasma centrale*; sin embargo, en México sólo se ha identificado la primera especie. En varios países del mundo *A. centrale* se utiliza como vacuna contra la anaplasmosis bovina. En nuestro país es considerada especie exótica y su uso como vacuna está prohibido. No obstante, es importante tener pruebas moleculares que detecten los patógenos circulando en el ganado que se moviliza dentro y hacia afuera del país. Este trabajo se basa en un análisis pangenómico de cepas de *A. marginale,* aisladas en México, y *A. centrale* para identificar genes únicos de especie. La secuencia de estos genes únicos se utilizó para diseñar oligonucleótidos que amplifican productos de PCR utilizando como templado DNA genómico extraído de sangre de animales en campo y de sangre congelada. La prueba amplifica un fragmento de las secuencias de *exoSbcC* de *A. centrale* y *biotin* de *A. marginale*, candidatos para la detección de estas especies.

Palabras Claves: *Anaplasmosis bovina, ganado, detección molecular, muestreo*

Abstract
The detection of pathogens is essential for the surveillance of diseases that could develop in cattle in our country. *Anaplasma marginale* and *Anaplasma centrale* can cause bovine anaplasmosis; however, only the first species has been identified in Mexico. In several countries of the world, *A. centrale* is used as a vaccine against bovine anaplasmosis. In our country, it is considered an exotic species, and its use as a vaccine is prohibited. However, it is crucial to have molecular tests detect pathogens circulating in cattle moving in and out of the country. This work is based on a pan-genomic analysis of *A. marginale* strains isolated in Mexico and *A. centrale* to identify species-unique genes. The sequence of these unique genes was used to design oligonucleotides that amplify PCR products using genomic DNA extracted from field animal blood and frozen blood as a template. The test amplifies a fragment of the sequences of *A. centrale exoSbcC* and *A. marginale biotin* genes which are candidates for detecting these species.

Key Words: *Bovine anaplasmosis, cattle, diagnostic tool, PCR, sampling*

Introduction

Bovine anaplasmosis occurs in tropical and subtropical areas of the world and is endemic in Mexico, Central and South America, and the Caribbean Islands [1]. This microorganism infects bovine erythrocytes producing anemia, hemolysis, fever, abortions, weight loss, and a rapid drop in milk production in cows [2]. In Mexico, as in other tropical regions of the world, bovine anaplasmosis is caused by *Anaplasma marginale*; however, *Anaplasma centrale*, a naturally attenuated species, is considered to cause mild anaplasmosis in cattle [3]. *A. centrale* is less virulent, and it was detected by Sir Arnold Theiler, and 100 years later, this live vaccine is still used in South Africa, Israel, South America, and Australia [4,5]. In México, this species has not been reported, and its use as a vaccine is not

permitted because it is considered an exotic species by the authorities. Additionally, this "vaccine strain" of *A. centrale* was used to generate the complete genome sequence in 2010 [6].
Biological transmission of *A. marginale* is mediated by ticks of the genus *Rhipicephalus microplus*, *Dermacentor* spp., and *Ixodes* spp, while *A. centrale* is transmitted transstadially by the tick *R. simus* [7]. On the other hand, transmission can also occur mechanically by blood inoculation, biting flies, and by the use of surgery equipment [1,8].

The genetic diversity of *Anaplasma* spp. strains vary from one geographic region to another. Because of this, the detection of the causal agent is a critical step for its control and decision-making. Therefore, detecting *A. marginale* and *A. centrale* is an effective way to detect the presence of these microorganisms in cattle. One of the most widely used molecular detection methods for *Anaplasma* spp. is the polymerase chain reaction (PCR). Currently, several genes are used in these methods as molecular markers for bacterial identification, including 16S rRNA, 23S rRNA, the intergenic region 16S-23S rRNA, *rpoB*, *recA*, *gyrB*, *dnaK*, *amoA*, *oxc*, *hitA*, gene *D*, citrate synthase, *rOmpA*, and *rOmpB*, to mention a few (Machado et al. 2016). Specifically, in *A. marginale*, several molecular markers have been used to analyze the genetic diversity of this pathogen, including major surface proteins (MSP) such as MSP1a, MSP4, and MSP5, which are encoded by single genes [5]. In the last years, several reports about the molecular characterization of *A. marginale* strains have been reported [9–11].

This work aims to develop a molecular test based on pangenomic studies to identify genes specific to species that allow differentiation in the presence of *A. marginale* or *A. centrale* in cattle blood samples. Since, in Mexico, *A. marginale* reaches a prevalence higher than 50% in endemic areas [12], it is essential to have a detection method that allows distinguishing between the *Anaplasma* species circulating in cattle to make decisions and avoid the spread of the disease.

Materials and Methods

Bioinformatic analysis

We performed a pangenomic analysis to identify the genes specific to each species with the Get Homologues program between the seven genomes of *A. marginale* reported in Mexico (Assembly numbers GCA_00888801305.1, GCA_008690265.1, GCA_003331125.1, GCA_00888801275.1, GCA_008274665.1, GCA_00888801275.1, GCA_008690255.1, GCA_00888801275.1) the *A. marginale* reference strain St. Maries (GCA_000011945.1), and the genome of *A. centrale* (GCA_000024505.1) reported in the Genbank database. The generated binary pangenomic matrix that ranks 1 and 0 for the presence and absence of genes, respectively, unique gene sequences in each genome were identified from the gene annotation obtained from the RAST server (https://rast.nmpdr.org/).

Oligonucleotides design

We designed gene-specific oligonucleotides for the selected genes for *A. marginale* and *A. centrale* using the Primer-Blast tool from the NCBI database (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with the following parameters: 50% GC content, a Tm of 60°C, an average length of 23 bp, and an average amplicon of 300 bp.

Genomic DNA extraction

We extracted the genomic DNA from five frozen blood samples of strains (Mex-01-001-01, Mex-31-096-01, Mex-15-099-01, Mex-17-017-01, Mex-28-037-02) of *A. marginale* from the Germplasm, Anaplasmosis Unit (CENID-SAI, INIFAP) and from the blood of isolates from Tapalpa, Jalisco, with the ReliaPrepTM Blood gDNA Miniprep System kit (Promega). Briefly, 200 µL of blood was added in a 1.5 mL Eppendorf tube mixed with 20 µL of Proteinase K to perform protein digestion in the sample, then 25 µL of Cell Lysis Buffer (CLD) was added to release the genetic material of the bacteria. Subsequently, it was mixed for 10 seconds to obtain a homogeneous mixture and incubated at 56°C for 10 minutes.

Once the incubation time had elapsed, the mixture was pipetted to an extraction column, and 250 µL of Blinding Buffer (BBA) was added and centrifuged the sample for 1 minute at 16,300 g. After centrifugation, the column was washed three times with 500 µL of Column Wash Solution (CWD) and centrifuged for 3 minutes at 16,300 g. The extraction column was run through a centrifuge for 3 minutes at 16,300 g. The extraction column was then run through with 500 µL of CWD and then transferred to a 1.5 mL Eppendorf tube; 80 µl of nuclease-free water was added and incubated for 15 minutes, followed by 1.5 minutes of centrifugation at 16,300 g. Genomic DNA was quantified spectrophotometrically (NABI, Microdigital), and its integrity was verified by electrophoresis in 1% agarose gel stained with ethidium bromide (10 mg/mL). The PCR reactions to detect *A. centrale* were performed in collaboration with Ph.D. Evangelina Primo (INTA, Argentina).

Molecular detection of A. marginale and A. centrale

We prepared an end-point PCR reaction by mixing 12.5 µL of Master Mix (Bioline), 1 µL (10 pmol/µL) of the forward oligonucleotide, 1 µL (10 pmol/µL) of the reverse oligonucleotide, 300 ng of genomic DNA, and nuclease-free water for a final volume of 25 µl. Amplification conditions were: initial denaturation 94°C, 3 minutes; one denaturation cycle 94°C, 30 seconds; one alignment cycle 60°C, 30 seconds; 30 elongation cycles 72°C, 30 seconds and one final elongation cycle 72°C, 10 minutes. We verified PCR product amplification in a 1% agarose gel electrophoresis with ethidium bromide (10 mg/mL).

PCR product purification and sequencing

We used the Wizard® SV Gel and PCR Clean-Up System kit (Promega) to purify the amplicons and sequenced them at the Synthesis and Sequencing Unit of the Institute of Biotechnology UNAM.

Results

Identification of the genes of A. marginale and A. centrale

Genes specific to each species were identified in the pangenomic matrix generated between each *A. marginale* and *A. centrale* strain genome. The genome annotation with the RAST server identified the protein sequence encoding each gene. Some of the unique genes selected from *A. centrale* were exonuclease SbcC (fig|6666666.325746.peg.70); pentapeptide repeat domain protein (fig|6666666.325746.peg.256); streptococcal hemagglutinin protein (fig|6666666.325746.peg.912); cytoplasmic axial filament protein CafA (fig|6666666.325746.peg.1064); and RecA (fig|6666666.325746.peg.1164). The single genes selected from *A. marginale* were biotin operon repressor/biotin-protein ligase (fig|6666666.341241.peg.401) and proline dehydrogenase (fig|6666666.341241.peg.574).

Oligonucleotide Synthesis

Seven pairs of oligonucleotides were synthesized, five pairs for genes of *A. centrale* and two pairs for genes of *A. marginale*. The oligonucleotide sequences, amplicon size, and Tm values are shown in Table 1.

Genomic DNA extraction

The quality of the extracted genomic DNA from five frozen blood samples of Mexican strains of *A. marginale* (Mex-01-001-01, Mex-31-096-01, Mex-15-099-01, Mex-17-017-01, Mex-28-037-02) from the Germplasm of the Anaplasmosis Unit (CENID-SAI, INIFAP) and from 30 blood samples from cattle of Tapalpa, Jalisco (isolates 0201, 0268, 0270, 1122, 1132, 1174, 1173, 1180, 2357,4728, 7377, 7391, 8382, 8376, 3118, 0062, 0125, 0225, 1162, 3855, 4395, 4403, 4696, 4705, 6790, 9861, 9883, 9887, 9893, 9924), is shown in Figure 1.

Amplification of the unique genes of *A. marginale*

We obtained amplicons corresponding to 220 bp fragments of the biotin operon repressor/biotin-protein ligase (biotin) gene and 234 bp fragments of the proline

Table 1. Sequences of oligonucleotide pairs synthesized for PCR amplification of the single genes of *A. marginale* (AM) and *A. centrale* (AC).

Name	Sequence (5'-3')	Tm (°C)	Amplicon size (pb)
ACExoSbcCFwd	CAAGGCACTGGGAGAAAAGCTAGA	60	267
ACExoSbcCRev	CCTAGTATCCCCAACTTCTCGC	60	267
ACRecAFwd	GAGAATGGCGGTGAGGGTTTTGAT	60	194
ACRecARev	CTCACACCCAACTTCCTGGCATAC	60	194
ACPentapFwd	GCCGTGTTGAACAGAGCGAAAATC	60	192
ACPentapRev	AGACCATATAGCCTCACTGGCGTT	60	192
ACStrepFwd	CCAGCGGCAATGTATCGAAAGAC	60	300
ACStrepRev	TTTGTACTCCAGCTCCAGCTTCCT	60	300
ACCitoFwd	CAACAGCGGTGTGTGAAAATGGA	60	223
ACCitoRev	CTTAACAATTAAGCGAGGGGGCGT	60	223
AMBiotinFwd	CCGGAGCTGAAGCTATCGAACTTG	60	220
AMBiotinRev	CAAGCACCATTGTCCATGGGTAT	60	220
AMProlinFwd	AAGCGCATGCAGGGGTTGTA	60	234
AMProlinRev	CTAAGCAGGGACTCCGCCA	60	234

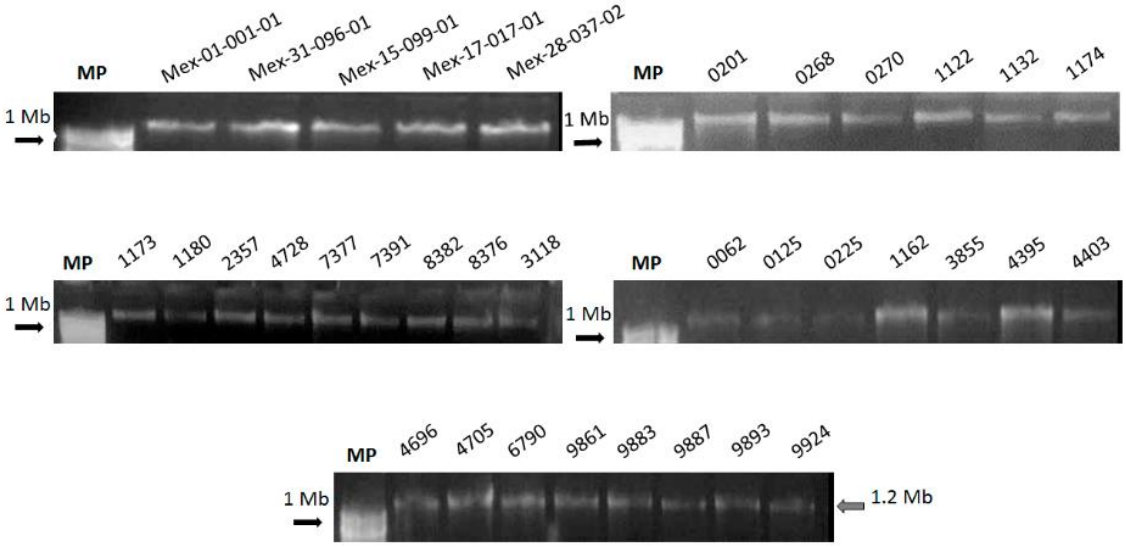

Figure 1. Mexican strains and isolates used for genomic DNA extraction. The genomic DNA was extracted from blood from Germplasm (CENID-SAI, INIFAP) and isolates from Jalisco, México.

dehydrogenase (prolin) gene of *A. marginale* with the corresponding oligonucleotides pair.

Of the two genes, we selected the gene biotin for further PCR amplification using DNA from isolates. The amplicons were observed in samples from blood from the Germplasm and isolates (Figure 2 and Table 2). Additionally, BLASTn bioinformatic analysis of the sequences reported in the electropherograms showed that the biotin operon repressor/biotin-protein ligase gene has an identity of 99.84% with the *A. marginale* strain Jaboticabal. In comparison, the identity of the proline dehydrogenase gene was 99.48% with the same strain.

Figure 2. Agarose-gel electrophoresis of PCR amplicons of A. marginale specific genes. The size of the *Biotin* and *Prolin* amplicons are 220 and 234 bp, respectively. The Mexican *A. marginale* strains resulted in positive for both genes. Only one amplicon for *Prolin* gen was observed in strain Mex-17-017-01. In *A. centrale*, none of the amplicons were observed.

Table 2. Results of PCR amplification with oligonucleotides targeting *A. marginale* gene *Biotin* in Mexican isolates

Sample	Result	Sample	Result
0201	Negative	0062	Positive
0268	Negative	0125	Positive
0270	Negative	0225	Positive
1122	Negative	1162	Positive
1132	Negative	3855	Positive
1173	Negative	4395	Positive
1180	Negative	4403	Positive
2357	Negative	4696	Positive
4728	Negative	4705	Positive
7377	Negative	6790	Positive
7391	Negative	9861	Positive
8382	Negative	9885	Positive
8376	Negative	9887	Positive
3118	Negative	9924	Positive
MQ water	Negative		

Amplification of the unique genes of *A. centrale*

No amplification of any of the five genes selected for *A. centrale* (exonuclease SbcC (Exo SbcC), pentapeptide repeat domain protein (Pentapep), streptococcal hemagglutinin protein (Strep), cytoplasmic axial filament protein CafA (Cito), and RecA) was observed in any of the samples tested (Table 3). We only observed amplicons of the expected sizes in the samples with *A. centrale* genomic DNA as template (Figure 3). The identity percentages obtained after BLASTn bioinformatic analysis of the electropherogram sequences showed that the percentage of identity for the Exo SbcC, Pentapep, Strep, Cito, and RecA genes is 99.55, 95.39, 98.04, 99.63, and 99.04%, respectively, with the *A. centrale* Israel strain.

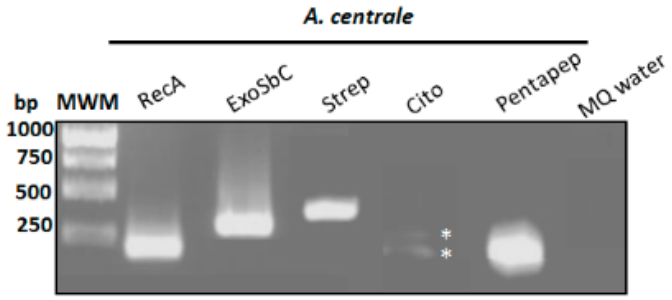

Figure 3. Agarose-gel electrophoresis of PCR amplicons of *A. centrale* specific genes. The size of the *RecA*, *ExoSbC*, *Strep*, *Cito*, and *Pentapep* amplicons observed are 194, 267, 300, 223, and 192 bp, respectively. The control reaction using MQ water was negative for all *A. centrale* genes.

Discussion

Bovine anaplasmosis, caused by *A. marginale*, has a wide distribution in Africa, Australia, Asia, and Latin America, generating economic losses. In Mexico and other countries, the development of molecular tests to detect *Anaplasma* spp. is a vital tool for a specific diagnosis and to initiate the corresponding treatments to control the disease. In order to develop a molecular test for the detection of *Anaplasma* spp., a selection of genes specific to *A. marginale* and *A. centrale* based on genomic analysis has been carried out to obtain a specific and effective detection method.

This analysis aimed to identify genes of *A. marginale* and *A. centrale* with potential as molecular markers for the detection of both bacteria from genomic DNA extracted from blood samples of cattle. We evaluated *A. marginale* strains from the Germplasm Bank (CENID-SAI) and bovine blood isolates from Tapalpa, Jalisco. In this work, we performed a genomic analysis to select specific *A. marginale* genes that are not in *A. centrale* genome and vice versa. Once this criterion was established, the genes *ExoSbcC*, *Pentap*, *RecA*, *Strep*, and *Cito* of *A. centrale* and *Biotin* and *Prolin* of *A. marginale* were considered as unique genes.

For *A. marginale*, we detected amplicons for the *Biotin* (220 bp) and *Prolin* (234 bp) genes in strains Mex-01-001-01, Mex-15-099-01, Mex-28-037-02, and, Mex31-096-01. The PCR reaction barely amplified both genes in strain Mex-17-017-01 (Figure 2), probably due to a low amount or to the degradation of the template DNA. The presence of *A. marginale* was also detected in the isolates from Tapalpa, Jalisco (Table 2). In *A. centrale* none of the genes were detected neither in Mexican strains nor isolates from Jalisco.

Table 3. Results of PCR amplification with oligonucleotides targeting *A. centrale* gene *ExoSbC* in Mexican isolates

Sample	Result	Sample	Result
0201	Negative	0062	Negative
0268	Negative	0125	Negative
1132	Negative	1162	Negative
1173	Negative	4403	Negative
2357	Negative	4696	Negative
4728	Negative	4705	Negative
7377	Negative	6790	Negative
8376	Negative	9861	Negative
1180	Negative	9893	Negative
0270	Negative	9924	Negative
1174	Negative	MQ water	Negative
8382	Negative		
3118	Negative		
7391	Negative		
1122	Negative		
0225	Negative		

A. centrale was detected with the primers *RecA*, *ExoSbcC*, *Strep*, *Pentapep*, and *Cito* (Figure 3) using DNA from *A. centrale* as template which suggest that these five genes could be used to detect this pathogen by molecular methods. On the contrary, no amplification product was observed in any Mexican strains or isolates (Table 3).

This work is a basic approach for detecting both species of *Anaplasma* in our country. Although endpoint, nested, and real-time PCR assays have already been developed elsewhere, they are based on genes from reference strains that are not necessarily genetically identical to those present in our country [13–15].

The use of PCR of different types in diagnosing *A. marginale* and *A. centrale* allows advances in diagnosis and the design of efficient detection methods and epidemiological studies [16–19].

On the other hand, to confirm that the amplicons corresponded to *Anaplasma* spp. sequences, they were sequenced and analyzed bioinformatically by BLASTn. It was observed that the amplified products in the Mexican strains and isolates have percentages of the identity of 99.84% with Brazilian strains reported in NCBI. Meanwhile, the identity of the sequences obtained by amplification of *A. centrale* showed percentages higher than 99% with the Israel strain. Because *A. centrale* is considered a less virulent species than *A. marginale* it has potential as a vaccine against anaplasmosis. However, there are studies on infections caused by *A. centrale*, such as the one in 2008, where 58 cases of bovine anaplasmosis caused by *A. centrale* were reported in Europe [20].

Using the PCR technique for identifying *A. centrale* has generated favorable results. Khumalo et al. (2016) used real-time PCR to identify and characterize *A. centrale* in buffaloes and cattle.

These first results obtained are of great importance for the anaplasmosis control programs developed in our country since we could implement new tools for detecting the disease. Although it is necessary to establish more analyses to detect *A. centrale*, using a more significant number of samples from different country regions and perform sensibility test to assess the minimal amount of genomic DNA necessary to detect the pathogen.

Finally, establishing a molecular test to detect *A. centrale* in our country is vital due to its close relationship with *A. marginale* and the continuous importation of cattle to our country from South America, where *A. centrale* is used as a vaccine, which increases the probability of finding this pathogen in the country.

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