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5 **ORIGINAL RESEARCH ARTICLE**

6 Integration of molecular testing to confirm the presence of 7 *Tritrichomonas foetus* in cattle from northwest Mexico

8 ***Integración de pruebas moleculares para confirmar la presencia de Tritrichomonas***
9 ***foetus en ganado del noroeste de México.***

10 ***Integração de testes moleculares para confirmar a presença de Tritrichomonas foetus em***
11 ***bovinos do noroeste do México.***

12

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Abstract

Background: Bovine trichomoniasis is a sexually transmitted disease caused by *Tritrichomonas foetus* that significantly impairs the reproductive health of cattle. While bulls are asymptomatic carriers of this protozoan, cows infected with *T. foetus* exhibit embryonic deaths, abortions, reproductive tract infections, or infertility. In Mexico, the first report of *T. foetus* was made more than 60 years ago; however, efforts to control this disease through diagnosis have been limited despite the negative impact on livestock farming. **Objective:** To confirm the presence of *T. foetus* in animals from northwest Mexico via molecular detection, sequencing, and phylogenetic analyses. **Methods:** Forty biological samples (smegma and mucopurulent vaginal secretion) were collected and analyzed by PCR technique to identify *T. foetus* using specific primers. To confirm homology and determine genetic relationships, sequencing and phylogenetic analyses were performed using amplicons obtained from *T. foetus*-positive animals. **Results:** The PCR technique identified *T. foetus* in smegma and in clinical cases of metritis. Sequences obtained from PCR products revealed a 99.65% of similarity and a bootstrap of 99% with isolates of *T. foetus* reported in China, Spain, and USA. Based on the sequencing results, five new Mexican isolates of *T. foetus* were published in NCBI. **Conclusion:** This is the first report confirming the presence of *T. foetus* in Mexico using sequencing and phylogenetic analyses from positive cattle samples. Our research effort represents the basis for developing alternative and accurate tests to diagnose *T. foetus* as part of the strategy to epidemiologically control bovine trichomoniasis in the country.

Keywords: *abortions; bull; cow; homology; infertility; phylogeny; smegma; trichomoniasis.*

Resumen

Antecedentes: La tricomoniasis bovina es una enfermedad de transmisión sexual causada por *Tritrichomonas foetus* y tiene un impacto significativo al afectar la salud reproductiva en hatos bovinos. El toro es el portador asintomático de este protozoario, pero en la vaca *T. foetus* puede ocasionar muerte embrionaria, aborto, infecciones del aparato reproductivo o infertilidad. En México, el primer reporte tiene más de 60 años; sin embargo, los esfuerzos para controlar esta enfermedad a través de su diagnóstico han sido escasos a pesar del impacto negativo a la ganadería. **Objetivo:** Confirmar la presencia de *T. foetus* mediante análisis de

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secuenciación y filogenia a partir de aislados moleculares obtenidos de animales positivos criados al noroeste de México. **Métodos:** Se colectaron 40 muestras biológicas (esmegma y contenido vaginal purulento) y se analizaron por la técnica de PCR para identificar *T. foetus* utilizando oligonucleótidos específicos. Para confirmar la homología y determinar las relaciones genéticas, se realizaron secuenciaciones y análisis filogenéticos utilizando amplicones obtenidos de animales positivos a *T. foetus*. **Resultados:** La técnica de PCR identifico *T. foetus* en esmegma y en los casos clínicos de metritis. Las secuencias obtenidas de los productos de PCR revelaron una identidad del 99.65% y un bootstrap del 99% con aislados reportados de China, España y EE. UU. Con base a los resultados de la secuenciación, se publicaron en NCBI cinco nuevos aislados mexicanos de *T. foetus.* **Conclusión:** Este es el primer trabajo que confirma la presencia de *T. foetus* en México mediante secuenciación y filogenia a partir de casos positivos en bovinos. Este estudio será la base para desarrollar pruebas alternativas y precisas para diagnosticar *T. foetus* como parte de la estrategia para controlar epidemiológicamente la *tricomoniasis* bovina en el país.

Palabras clave: *abortos; esmegma; filogenia; homología; infertilidad; toros; tricomoniasis; vaca.*

Resumo

Antecedentes: A tricomoníase bovina é uma doença sexualmente transmissível pelo *Tritrichomonas foetus* e tem um impacto significativo na saúde reprodutiva dos rebanhos bovinos. O touro é o portador assintomático desse protozoário, mas na vaca o T. foetus pode causar morte embrionária, aborto, infecções do trato reprodutivo ou infertilidade. No México, o primeiro relato tem mais de 60 anos; entretanto, os esforços para controlar esta doença através do diagnóstico têm sido limitados, apesar do impacto negativo na pecuária. **Objetivo:** Confirmar a presença de *T. foetus* por meio de sequenciamento e análise de filogenia de isolados moleculares obtidos de animais positivos criados no noroeste do México. **Métodos:** 40 amostras biológicas (esmegma e conteúdo vaginal purulento) foram coletadas e analisadas pela técnica de PCR para identificar *T. foetus* usando primers específicos. Para confirmar a homologia e determinar as relações genéticas, o sequenciamento e a análise filogenética foram realizados usando amplicons obtidos de animais positivos para T. foetus. **Resultados:** A técnica de PCR identificou o *T. foetus* no esmegma e em casos clínicos de metrite. As

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sequências obtidas dos produtos de PCR revelaram uma identidade de 99,65% de similaridade e um bootstrap de 99% com isolados relatados na China, Espanha e EUA. Com base nos resultados do sequenciamento, cinco novos isolados mexicanos de *T. foetus* foram publicados no NCBI. **Conclusões:** Este é o primeiro trabalho que confirma a presença de *T. foetus* no México por meio de sequenciamento e filogenia de casos positivos em bovinos. Esse trabalho será a base para o desenvolvimento de testes alternativos e precisos para diagnosticar o *T. foetus* como parte da estratégia de controle epidemiológico da tricomoníase bovina no país

Palavras-chave: *abortos; filogenia; homologia; infertilidade; smegma; touros; tricomoníase; vaca.*

Introduction

Bovine trichomoniasis is a venereal disease caused by *Tritrichomonas foetus*, a flagellated protozoan that lodges in the cavities of gastrointestinal and reproductive tracts of infected animals (Marlene *et al.,* 2017*)*. In bulls, *T. foetus* is found on the epithelial surface of the penis and preputial crypts, and although it is capable of producing nodules in the zone, typically does not produce clinical signs causing that males act as asymptomatic carriers (Parsonson, 1976; Mohammed *et al.,* 2023). Conversely, infected females do show clinical signs including vaginitis, endometritis, birth of weak calves, fetal maceration, and abortions; affecting calf production and causing significant economic losses to producers (Martínez *et al.,* 2023).

In Mexico, the first report of bovine trichomoniasis was made more than 60 years ago (Cuevas, 1967). In recent years, the importation of bulls to Mexico from other countries where *T. foetus* is endemic, along with the increase in the number of cases with clinical signs suggestive to the parasite has generated concern among animal health professionals and producers, giving rise to the need for diagnostic tools that allow the control of this disease. A study conducted in Chihuahua, a northern Mexican state, using a commercial culture system for PCR as diagnostic method, reported that 21.8% of the sampled bulls were positive to *T. foetus*, while 60 to 65% of the evaluated herds had at least one positive animal (Ramírez,

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111 2017). In turn, Villareal (2020) employed classic microscopy and reported that 36% of the
112 cows and 14.3% of the bulls that were sampled in Veracruz (an eastern state of Mexico) were
113 positive to this protozoan.

114 The standard diagnostic method for trichomoniasis is the microscopic identification of the
115 protozoan from diverse biological samples like preputial washings, uterine fluid, and
116 cervicovaginal secretions. For this method, is important to highlight that samples need to be
117 cultured in a medium suitable for the replication and growth of *T. foetus* (Dabrowska *et al.,*
118 2021). Limitations of this technique include variations due to sampling conditions,
119 sensitivity, specificity, and overall accuracy. Moreover, the human factor must also be
120 considered since trained personnel are required to perform an accurate diagnosis. To
121 overcome such challenges, molecular methods have been developed in cattle for the specific
122 detection of *T. foetus*. In the last years, the polymerase chain reaction (PCR) has become one
123 the most popular method given it can exclude false positive cultures (WHOA, 2018),
124 therefore it remains a reliable test that can be used as a reference for the development of other
125 molecular assays.

126 Controlling trichomoniasis in Mexico is challenging due to the scarcity of data related to the
127 prevalence of the disease and the potential severity of its impact. Obtaining such information
128 relies on efficient diagnostic tests capable of accurately identifying the etiological agent. So,
129 the effort in the development and standardization of alternative diagnostic tests for *T. foetus*
130 has recently begun in the country. Importantly, for the any test standardization it is required
131 to ensure that the genetic material analyzed corresponds specifically to the pathogen of
132 interest. In this regard, sequencing and phylogenetic analysis can contribute to the
133 confirmation of the identity and phylogeny of the genetic material identified as positive for
134 *T. foetus*. Therefore, the aim of the present study was to confirm the presence of *T. foetus* by
135 sequencing and phylogenetic analyses of molecular isolates obtained from positive animals
136 raised at the northwest of Mexico.

137 **Materials and methods**

138 *Ethical considerations*

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No animals were injured while handled or during the sample collection procedure. The study protocol for this project was reviewed and approved (report No 2024-04) by the Animal Welfare and Research Ethics Committee of the Instituto Tecnologico de Sonora.

Study location and animal population

The study was conducted in the northwestern region of Mexico. A total of forty animals from eight herds were included in the study. Specifically, 30 bulls of different breeds with a history of at least two breeding seasons, as well as 10 adult cows with previous records of abortions.

Sample collection

Smegma samples were collected from the bulls' preputial cavities (Dabrowska *et al.,* 2021). First, each bull was immobilized in a cattle chute, then upon access to the bull's prepuce, excess dirt was removed with disposable paper. Subsequently, the zone was disinfected with 2% iodine while the hairs of the distal end of the prepuce were trimmed, and excess moisture was removed with paper. After cleaning and disinfection, an external massage was applied for two to three minutes to relax the preputial mucosa. A plastic infusion pipette with a blunt tip was introduced into the prepuce up to the sigmoid flexure, where 20 ml of phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO) solution were deposited while stirring by hand. Suction was then applied with the syringe while the pipette was gently moved up and down along the preputial mucosa, recovering the largest amount of PBS with preputial cellular tissue. Finally, the sample was placed in a sterile disposable 20 ml test tube and labeled with the animal's information (Oyhenart, 2018).

In cases of abortion (no more than 14 days after the abortion event), purulent vaginal secretion samples were obtained from the uterine content of cows with clinical metritis. y High viscosity samples were diluted with 10 ml of PBS and centrifuged at 2200 *g* for 15 min. The supernatant was removed, and the pellet was resuspended again in a volume of 1 ml. Finally, 200 µl were used for DNA extraction, and the rest was frozen at -60 °C until further use.

Automated nucleic acid extraction

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169 Diluted smegma and purulent vaginal secretion samples were first homogenized by vortexing
170 and inversion. DNA extractions were then performed using the taco™ (taco™ Nucleic
171 Acid Automatic Extraction System, taco™; GeneReach USA) with the taco™ DNA/RNA
172 Extraction Kit, following the manufacturer's protocol. The extracted nucleic acid was
173 quantified with UV spectrophotometry (BioSpect-Nano, Shimadzu®). The integrity of the
174 extracted DNA was verified by electrophoresis in a 1.5% agarose gel stained with ethidium
175 bromide. All extractions were stored in sterile 1.5 ml vials at -20 °C to be subsequently
176 processed for the PCR technique.
177
178 *PCR detection of T. Foetus in biological samples*
179 DNA samples were analyzed by PCR using the primer set TFR3: 5'-
180 CGGGTCTTCCTATATGAGACAGAACC-3' and TFR4: 5'-CGGGTCTTC
181 CTATATGAGACAGAACCGGAGCTGAATG-3', amplifying a 347 bp region of the gene
182 encoding the 5.8S rRNA, as well as the ITS1 and ITS2 regions of *T. foetus* (Felleisen *et al*.,
183 1998). For the reactions, the pre-loaded GoTaq® Flexi DNA Polymerase PCR kit (Promega®)
184 was used, containing Green GoTaq®, which serves as a reaction buffer and gel loading
185 solution, allowing reactions to be loaded directly for rapid and efficient analysis. Reactions
186 were performed in a final volume of 25 µL, with a concentration of 1X Green GoTaq Buffer
187 5x, 1.5 mM MgCl2, 0.2 mM for each dNTP, 0.4 µM of each primer, 1.25 µl. of GoTaq DNA
188 Polymerase, 5 µl of DNA and nuclease-free H2O at 25 µl. Analytical conditions for PCR
189 were 1 cycle of 95 °C for 3 min, 32 cycles of: 95 °C for 15 s, 62 °C for 30 s, and 72 °C for
190 30 s, and a final extension step of 72 °C for 5 min. The product was identified on a 1.5%
191 agarose gel with ethidium bromide, considering positive bands with the size of the agent
192 amplicon.
193
194 *Phylogenetic analysis*
195 Five positive PCR products were selected and sequenced using the Sanger dideoxy
196 sequencing method at the Institute of Biotechnology of the Universidad Nacional Autonoma
197 de Mexico (UNAM). The sequences obtained were analyzed using the MEGA 11 software
198 (Tamura *et al*., 2021) employing the Clustal W and UPGMA alignment methods, based on
199 multiple sequence alignment analysis. A phylogenetic analysis was performed to determine

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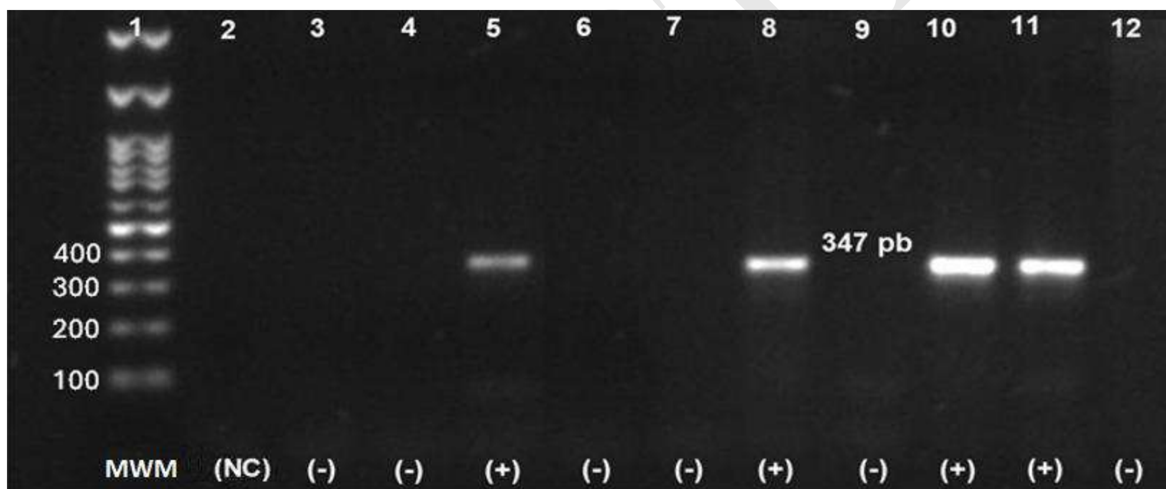
200 the identity between Mexican local *T. foetus* isolates (MX-TFI) and *T. foetus* isolates
201 registered within NCBI (National Center of Biotechnology Information) through BLAST.
202 Finally, all local isolates were registered in GenBank-NCBI.
203
204 **Results**
205 With an average quantification of 58.32 ng/µl according to spectrophotometry and a purity
206 of 1.85 in the 260/280 OD ratio, the electrophoresis results showed good integrity of the
207 extracted DNA and confirmed the presence of adequate amounts of quality DNA free of
208 contaminants such as guanidine, phenols, chaotropic salts or carbohydrates. As per PCR
209 detection, nine out of the 30 bulls (30% of the males) and 2 out of the 10 cows (20% of
210 females) were positive for *T. foetus*. In the positive samples, the specific 347 bp band
211 corresponding to the ribosomal DNA region of *T. foetus* was observed (Figure 1).



212
213 **Figure 1.** Representative electrophoresis of a 1.5% agarose gel treated with ethidium
214 bromide. Lane 1: Molecular weight marker (MWM) BH 100bp DNA Ladder RTU. Lane 2:
215 Negative control (NC). Lanes 5, 8, 10, 11: Positive. Lanes 3, 4, 6, 7, 9, 12: Negative.
216
217 Data generated from sequencing of purified PCR amplicons were analyzed and named for
218 publication within the NCBI database as new Mexican isolates of *T. foetus*: *CEAL-1*
219 (PQ474277), *CEAL-2* (PQ474278), *CEAL-3* (PQ474279), *CEAL-4* (PQ474280), and *CEAL-*
220 *5* (PQ474281). According to NCBI-BLASTn, the alignments of the nucleotide sequences of
221 the MX-TFI and the isolates from GenBank revealed a similarity of 99.65% to isolates
222 previously reported in China, Spain, and the United States. This variation in the percentage

of similarity is the result of a deletion of a thymine in the Mexican sequences unlike all the isolates reported in NCBI-BLASTn and aligned in the MEGA 11 software (Figure 2).

Figure 2. Alignment of ribosomal small subunit (SSU rRNA) sequences from different *T. foetus* isolates using Clustal W. The yellow rectangle marks the deletion of Mexican *Tritrichomonas foetus* isolates (MX-TFI).

The phylogenetic tree compares *T. foetus* sequences isolated from different geographic regions and hosts, showing two main groups with a high level of bootstrap support (99%), indicating confidence in the grouping of these clades (Figure 3). The first five isolates at the top (*CEAL-1* to *CEAL-5*) correspond to Mexican *T. foetus* isolates (MX-TFI) and the 99% of support demonstrates the close relationship between them. These isolates are more closely related to other *T. foetus* from countries such as China (OP856640.1), Spain (MK770850.1), and the United States (MK250821.1), forming a subgroup within the same clade. Another strong cluster with 99% bootstrap support includes isolates from Brazil (KX267765.1, HM400076.1, KU680816.1) and Turkey (HM490937.1). These are more distant from the MX-TFI and form a cluster of their own, which could suggest a geographic or evolutionary divergence. Isolates from different regions such as Namibia (KX425878.1), Australia (MW322817.1), Japan (LC054294.1) and the United States (AY349189.1) were observed, forming independent branches in the tree, but all within the same major clade. Finally, at the bottom of the tree, important reference isolates were included, such as *T. foetus* USA (M81842.1) and the *T. foetus* isolate from Iran (OL455037.1), showing that these isolates are

more distant from the others, which could reflect more marked genetic differences among these and the other isolates.

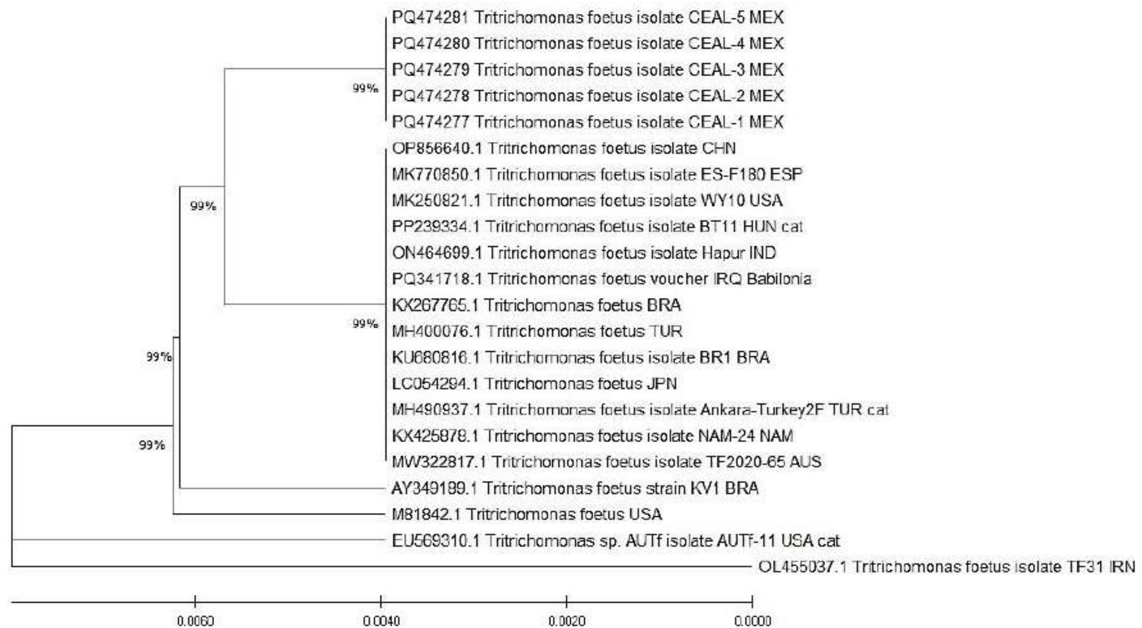


Figure 3. Phylogenetic tree analysis of Mexican *Tritrichomonas foetus* isolates (MX-TFI) and *Tritrichomonas foetus* isolates obtained from NCBI-BLAST.

Discussion

In molecular studies, the quality and quantity of extracted DNA are crucial factors for PCR amplification testing and sequencing. The quantification obtained by spectrophotometry was 58.32 ng/µl, suitable for most molecular applications since minimum concentrations of 50 ng/µl are required for PCR (Ahn *et al.,* 1996). The 260/280 OD ratio was 1.85, confirming good purity with a low presence of contaminants, according to the Microbiological Biosafety Laboratory Manual (SENASICA, 2014). These results are similar to those reported by Gharban (2023), who obtained 45.7 ng/µl and purity of 1.73 in a study using purulent vaginal secretion from cows having abortions. The slight difference in DNA concentration may be due to variations in the extraction protocol, sample type, or specific study conditions. However, both studies highlighted that adequate DNA quality was achieved, which ensured the success of subsequent molecular applications.

The comparison of the incidence and detection results of *T. foetus* and the scarcity of data available in different states of Mexico over time, show a worrying trend in the lack of attention to establish the incidence or prevalence of this disease in national livestock. In our study, 30% of the bulls and 20% of the cows were detected as PCR positive, indicating a considerable presence of the disease in the region. This reflects consistency with the findings of Cuevas (1967), who reported the disease in dairy cattle from the Valley of Mexico more than 60 years ago, which suggests that *T. foetus* has been a silent and persistent problem in the industry. More recently, Ramírez (2017) reported a prevalence of 21.8% in sampled bulls, and 60-65% of the farms evaluated in Chihuahua had at least one positive animal. This figure, although lower than the prevalence observed in our study, indicates that trichomoniasis continues to be a significant challenge in the northwest of Mexico. Nonetheless, evidence that this could represent a nationwide problem exists since in Veracruz (eastern Mexico), a prevalence of 36% in cows and 14.3% in bulls was reported (Villareal, 2020). These findings underline the importance of implementing effective diagnostic and control strategies. As bulls are moved from different regions and countries, the risk of disease spread increases, making early and accurate diagnosis essential. The use of molecular techniques such as PCR has improved detection capacity, allowing infections to be identified at earlier stages and contributing to more effective management of reproductive health in livestock.

Furthermore, phylogenetic studies of *T. foetus* can show relevant information about the genetic variability and potential relationships between different strains isolated from different hosts and geographical regions. In our phylogenetic analysis, some strains into two large clades were observed, indicating a close relationship between the Mexican isolates and strains from countries such as China, Spain, and the United States. This strong grouping suggests that the isolates from Mexico share a recent common ancestor with strains from those countries and could imply transmission routes or genetic exchange between populations.

On the other hand, Reinmann *et al*. (2012), Šlapeta *et al*. (2012), and Sun *et al*. (2012) indicated that, despite the moderate genetic distinction between feline, bovine, and porcine genotypes of *T. foetus*, these might represent a single species. Similarly, Gharban (2023)

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294 reported that *T. foetus* genotypes in cattle and cats show a degree of homogeneity, supporting
295 the idea of close identity between isolates from different host species. This study also
296 suggests that host exchange might be a real phenomenon, although cases of interspecies
297 transmission in natural settings have not yet been documented. Conversely, a multilocus
298 analysis by Pedraza-Díaz *et al*. (2019) identified more marked genetic differences between
299 *T. foetus* from cats and cattle, suggesting that, although there are similarities, there may also
300 be genetic differentiations that reflect adaptations to their respective hosts.
301
302 In our phylogenetic tree, independent branches of isolates from Namibia, Australia, Japan,
303 and the United States were highlighted, which could suggest a scenario in which different
304 populations of *T. foetus* have evolved in isolation. This contrasts with the close relationship
305 observed between Mexican isolates and other strains from the American and European
306 continents, which could indicate greater connectivity or genetic exchange in those regions.
307 Finally, reference isolates of *T. foetus* such as the one from the United States and the one
308 from Iran, show themselves more distant from the rest, suggesting that there are significant
309 genetic differences that may be relevant for the development of diagnostics and treatments
310 in different cattle populations. This pattern of genetic variability in *T. foetus* underscores the
311 need for continued surveillance and phylogenetic studies to better understand the
312 epidemiology of the disease and its impact on animal health. The similarities and differences
313 observed between our results and those presented by Marlene *et al*. (2017) and Abdel-Glil *et*
314 *al*. (2024) who presented a draft and assembly of the complete genome of a bovine strain,
315 highlight the importance of continuing to investigate the genetic variability and epidemiology
316 of this protozoan in a global context.
317
318 Although available in Mexico, some reliable molecular techniques such as PCR can be
319 expensive to employ on a large scale because their use depends on the importation of reagents
320 that in some cases cannot be replaced due to prior standardization of the test. In addition, the
321 cost of the test is also conditioned by the need for sophisticated equipment and infrastructure.
322 In this sense, some producers have even opted to send samples to other countries (e.g. USA)
323 for analysis; however, not all cattle owners can afford such an expense. The present study
324 will allow the development of an alternative test to PCR. That is, the sequences of Mexican

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325 isolates will provide the genetic basis for the design of a simpler, less expensive, even
326 portable molecular test. This is essential for the development of effective control strategies,
327 the prevention of outbreaks and the improvement of reproductive health in livestock, thus
328 contributing to the sustainability and profitability of the national livestock industry.
329

330 Conclusion

331 In this study, the presence of *T. foetus* was confirmed in the northwest of Mexico, and the
332 genetic structure of the pathogen was characterized. This is the first sequencing and
333 phylogenetic analysis of *T. foetus* in the country, providing a solid basis for accurate
334 identification of the disease and characterization of the genetic variability of local strains
335 compared to international isolates. The Mexican strains identified in this study represent a
336 crucial step to understand the epidemiology and new diagnostic alternatives for the bovine
337 trichomoniasis in Mexico.

338 Declarations

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346 *Conflicts of interest*

347 The authors declare they have not conflicts of interest related to this report.

348 *Authors contribution*

349 Conceptualization and design of the study: Flores-Encinas, Aragón-López, Leyva-Corona.
350 Wrote the first draft: Flores-Encinas, Aragón-López, Leyva-Corona. Laboratory procedures:
351 Flores-Encinas, Aragón-López, Figueroa-López. Edited and review the manuscript: All
352 authors. Sample collection: Flores-Encinas, Aragón-López, Leyva-Corona, Torres-Simental.

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353 Search for funding: Leyva-Corona, Morales-Pablos. All authors were in agree with the last
354 edition of the manuscript.

355 *Use of artificial intelligence (AI)*

356 The authors declare that during the preparation of this work no AI or AI-assisted technologies
357 were used.

358

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