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Original

Phylogenetic analysis of cysteine-protease sequences of *Tritrichomonas foetus*: crossing the species barrier?

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ABSTRACT

Objectives. To analyze the cysteine-protease (CP) gene sequences of *Tritrichomonas* spp. reported in GenBank, and develop a phylogenetic analysis to help clarify the possibility of transmission between species. **Materials and Methods.** The CP sequences of *Tritrichomonas* spp. available in GenBank were analyzed and aligned to identify polymorphic sites. The best nucleotide substitution model was determined, and phylogenetic trees were constructed for each gene, using *Trichomonas vaginalis* as an outgroup. Besides, tandem sequences of each reported pathogen were constructed to build phylogenetic trees with higher branch strength. Finally, the evolutionary divergence of the tandem repeat sequences was estimated to obtain more conclusive results. **Results.** The phylogenetic analysis showed the relationship that may exist between porcine *T. suis* and bovine *T. foetus*. The feline and bovine *T. foetus* sequences were found in separate groups; however, feline *T. foetus* was similar to that of bovines from Namibia. The proximity of human *T. foetus* to bovine and porcine *T. foetus* was verified. **Conclusions.** Phylogenetic analysis of CP sequences in different species of *Tritrichomonas* spp., identified relationships between bovine, porcine and human *Tritrichomonas*, but also between some isolated feline sequences with those of bovines, suggesting a possibility of barrier crossing between species.

Keywords: Cattle; protozoan; parasite; tritrichomoniasis (*Source: MeSH*).

RESUMEN

Objetivos. Realizar un análisis de las secuencias de los genes de las cisteína-proteasas (CP) de *Tritrichomonas* spp. reportadas en GenBank con el fin de desarrollar un análisis filogenético que ayude a esclarecer la posibilidad de transmisión entre especies. **Materiales y métodos**. Se realizó el análisis de las secuencias de CP de *Tritrichomonas* spp. disponibles en GenBank. Las secuencias halladas fueron alineadas, identificando sitios polimórficos. Se determinó el mejor modelo de sustitución nucleotídica y se construyeron árboles filogenéticos por cada gen, usando como grupo

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externo, *Trichomonas vaginalis*. Además, se construyeron secuencias en tándem de cada patógeno reportado, para construir árboles filogenéticos de mayor fortaleza en las ramas. Finalmente, se estimó la divergencia evolutiva de las secuencias en tándem. **Resultados.** El análisis filogenético evidenció la relación que puede existir entre *T. suis* porcino y *T. foetus* bovino. Las secuencias de *T. foetus* felino y bovino se encontraron en grupos separados; sin embargo, las de *T. foetus* felino fueron similares a las de bovinos de Namibia. También se pudo evidenciar la cercanía de *T. foetus* humano con *T. foetus* bovino y porcino. **Conclusiones.** El análisis filogenético de las secuencias de CP en diferentes especies de *Tritrichomonas* spp. permitió identificar relaciones entre las Tritrichomonas de bovinos, porcinos y humanos, pero también entre algunas secuencias aisladas de felinos con las de bovinos de Namibia, lo que sugiere la posibilidad del paso de la barrera entre especies.

Palabras Clave: Ganado; protozoarios; parásito; tritrichomoniasis (*Fuente: MeSH*).

INTRODUCTION

Tritrichomoniasis is an infection caused by the protozoan parasite *Tritrichomonas* spp., which belongs to the order *Trichomonida,* family *Trichomonidade*, and class *Parabasalia* (1). Tritrichomoniasis are common infections in the reproductive tract in bovines and is transmitted mainly through the genital route, resulting in embryonic death, infertility and abortions (2). In pigs, this parasite can invade the stomach, cecum and nasal cavity (3). Recently, a study indicated that it causes infections in the intestinal tract in cats, specifically in the lumen and crypt of the colonic mucosa epithelium, inducing cytopathic effects in epithelial cells causing chronic diarrhoea that is difficult to treat (4). Cysteine-proteases (CP), recognized as cytotoxicity mediators, provide the adherence and colonization of *Tritrichomonas foetus* to tissue, as well as its pathogenicity (5). This protozoan expresses a large number of genes associated with virulence or its metabolism, but some are highly conserved. For example, internal transcribed spacer (ITS) regions 1 and 2 are highly conserved, so they are not useful when performing a phylogenetic analysis and understanding the variation between species that may be associated with the host. For this reason, it is increasingly important to use more variable genes, as is the case of CP. Recently, CP2 has been stated as the most variable and adequate for performing phylogenetic analyzes (1).

Tritrichomonas spp. isolated from pigs has shown controversy regarding its identification. It was previously classified as *T. suis*; however, genetic and phylogenetic studies showed that the protozoan *T. suis* is the same bovine *T. foetus*, allowing its reclassification to the porcine *T. foetus* genotype (6). There are reports of opportunistic infections by *T. foetus* in immunodeppressed or immunosuppressed humans, allowing the colonization of *T. foetus* in the respiratory system (7). Nevertheless, the infection route and genotype of these parasites according to their host is still unclear (8)

Hence, the phylogenetic analysis could provide valuable information on the variations of the parasite and its relationship with the host. Accordingly, the aim of this study was to carry out a gene sequence analysis of cysteine-proteases of *Tritrichomonas* spp. reported in GenBank, to develop a phylogenetic analysis that helps clarify the possibility of transmission between species.

MATERIALS AND METHODS

Cysteine-protease sequences of *Tritrichomonas* spp. A search was carried out in GenBank using the words cysteine-protease and *Tritrichomonas* spp., obtaining reported sequences for different types of cysteine-proteases (CP1, CP2, and CP4). Subsequently, information on the geographic region and the species from which these were isolated was recorded. For this analysis, only the types of cysteine-proteases with *Tritrichomonas* spp. sequences that were also present in *Trichomonas vaginalis* were selected as an outgroup. Finally, 70 sequences were available for the analyzes, of which 18 belonged to CP1, 33 to CP2, and 19 were included in CP4. Additionally, a sequence of CP1, CP2, and CP4 of *Trichomonas vaginalis* (outgroup) were included.

Edition and phylogenetic analysis of sequences. The selected sequences were edited using the Molecular Evolutionary Genetics Analysis program v.10.1.8 (MEGA v.10.1.8.), and an alignment for each of the cysteine-proteases

(CP1, CP2, and CP4) reported in the National Center for Biotechnology Information (NCBI) employing the MUSCLE algorithm of the MEGA program v.10.1.8 was performed. From this alignment, the edition and a descriptive analysis of the sequences were carried out, registering the statistics related to the variable and conserved regions, and the best nucleotide substitution model was identified using the lowest value, according to the Bayesian information criterion (BIC) estimated using the MEGA program v.10.1.8.

The phylogenetic analysis was performed with Bootstrap through maximum likelihood using the nucleotide sequences of each cysteine-protease (CP1, CP2, and CP4). The best nucleotide substitution model obtained in each case was used, and information of the origin country and the species from which it was isolated was recorded to search for possible association patterns. 1000 bootstrap repetitions were used, and values higher than 70% were considered significant.

Construction of tandem sequences. From the previous information, cysteine-protease (CP1, CP2, and CP4) tandem sequences of the reported *Tritrichomonas* spp., and employing *Trichomonas vaginalis* as an outgroup were constructed, considering the origin country and the animal species from which they were isolated. A phylogenetic analysis was performed with Bootstrap through maximum likelihood with the K2 substitution model together with the discrete range distribution (K2+G) and a Bootstrap with 1,000 replications. Finally, the evolutionary divergence of the tandem sequences was estimated with the MEGA v.10.1.8 program.

Ethical aspects. In this article, an analysis of reported information was carried out, and no experimental procedures with animals were performed. The Bioethics Committee of Universidad Tecnológica de Pereira - CBE-UTP [code CBE-SYR-162016] approved this study.

RESULTS

The reported sequences of interest were three types of *Tritrichomonas* spp. cysteine-proteases isolated from different countries on four continents and different hosts, such as cattle, pigs, felines, non-human primates, and humans (Table 1). The sequences reported for Latin America were mainly obtained from bovines, and no cat sequences were found for this region. The records and access codes in GenBank for each sequence are presented in Table 1.

Table 1. Listing of sequences of cysteine-proteases of *Tritrichomonas* spp. reported in GenBank, with information on the species, animal host of the isolate, origin country, and access code in GenBank.

Gene	N	Species	Host	Country	GenBank Access Code
CP1	18	*T.suis* *T. foetus* *T. mobilensis* *Trichomonas vaginalis*	Porcine, Feline,Bovine, Saimiri boliviensis, Homo sapiens Human	Argentina, Australia, Bolivia, Czech Republic Germany, Japan, Namibia, USA, Switzerland. Unated Kingdom	JX648147.1, JX187024.1, JX648146.1, JX648148.1, JX187017.1, JX187015.1, JX187016.1, JX187014.1, JX187013.1, JX187018.1, JX187020.1, JX187019.1, JX187022.1, JX187021.1, KX425904.1, KX425905.1, LC054290.1, LC054281.1. X77218.1
CP2	33	*T.suis* *T. foetus* *T. mobilensis*	Porcine, Feline, Bovine, Saimiri boliviensis Homo sapiens	Argentina, Australia, Bolivia, Czech Republic, Germany, Japan, Namibia, USA, Switzerland	JX187040.1, JX648149.1, JX648150.1, JX648151.1, KP012652.1, JX187026.1, JX187027.1, JX187028.1, JX187029.1, JX187030.1, JX187031.1, JX187032.1, JX187033.1, JX187035.1, JX187036.1, JX187038.1, JX187039.1, JX187040.1, KX425891.1,KX425892.1, KX425893.1, KX425894.1, KX425895.1, KX425896.1, KX425897.1, KX425898.1, KX425899.1, KX425900.1, KX425901.1, KX425902.1, KX425903.1, LC054282.1, LC054291.1 X77219.1
		Trichomonas vaginalis	Human	Unated Kingdom	
CP4	19	*T.suis* *T. foetus* *T. mobilensis* *Trichomonas vaginalis*	Porcine, Feline, Bovine, Saimiri boliviensis Homo sapiens Human	Argentina, Australia, Bolivia, Czech Republic, Germany, Japan, Namibia, USA, Switzerland Unated Kingdom	JX648154.1, JX648153.1, JX187052.1, KP012653.1, JX187046.1, JX187045.1, JX187044.1, JX187043.1, JX187041.1, JX187042.1, KX425907.1, KX425906.1, JX187051.1, JX187050.1, JX187049.1, JX187048.1, JX187047.1, JX187053.1, LC054283.1 X77221.1

From the descriptive analysis of the sequence alignment of *Tritrichomonas* spp., it was evident that the one for CP4 showed the least variable sites (2), and the CP1 and CP2 sequences exhibited the most variable sites (4 and 28, respectively). The most extended alignment was found in the CP2 sequence with 669 nucleotides, and the shortest was observed in CP4 with 273 nucleotides.

The highest guanine-cytosine (G-C) percentage was found in the CP4 sequence with a range between 20.17% and 24.55%, and the lowest value was found in CP1 with values between 21.77% and 14.79%. The tandem sequences had a size of 2,283 nucleotides with 48 variable sites, and most of these (2,235) were conserved; further, a G-C percentage between 22.60 to 21.24 was recorded (Table 2). Overall, the best nucleotide substitution model was Kimura 2-parameters (K2); however, in the case of the CP2 sequences, parameter I (K2+I), which assumes that a certain fraction of the sites is evolutionarily invariant, was included. Furthermore, the tandem sequence used the G parameters (K2+G) that employed a discrete Gamma distribution to model non-uniform evolutionary rates.

From the best substitution model reports, the phylogenetic analysis was carried out, revealing a very similar topology of the different proteases evaluated in the constructed trees, with a clear separation of *Trichomonas vaginalis* from the rest of the *Tritrichomonas* spp. analyzed. Additionally, a grouping between the feline *Tritrichomonas foetus* in clades different from those of bovines, pigs and humans was identified. The grouping of the *T. foetus* of bovines from Namibia in the clade of *T. foetus* of felines, and very close to them, *T. mobiliensis*, draws attention, as bovines are the most susceptible group (Figure 1).

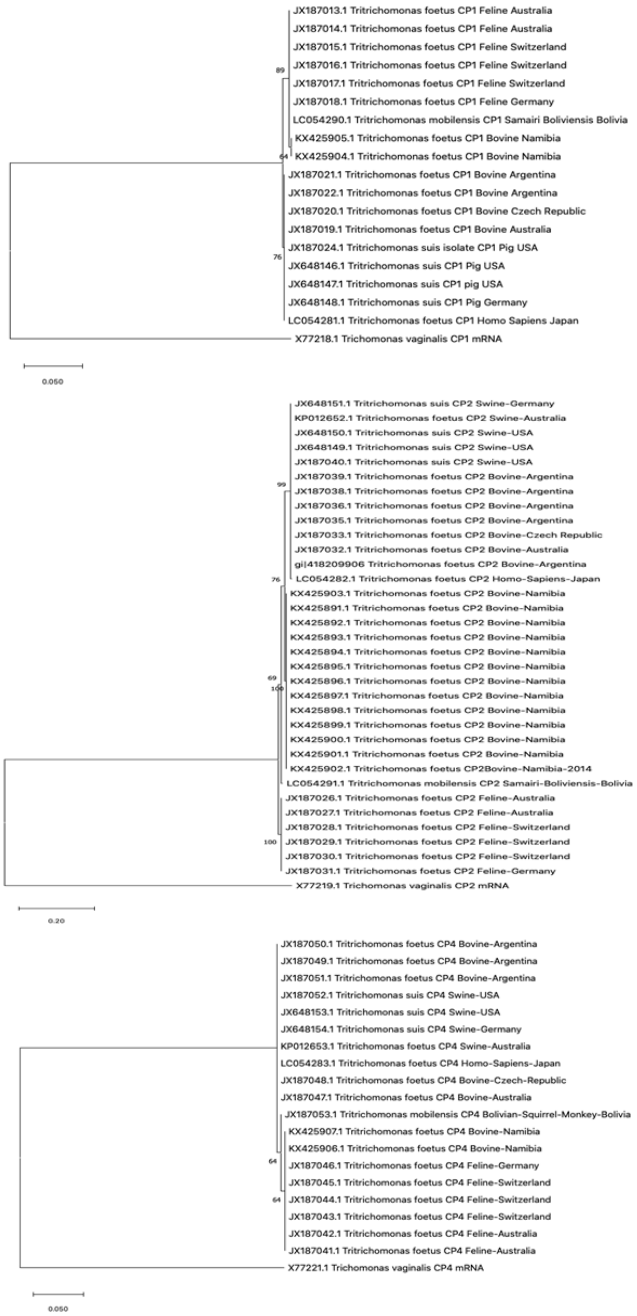

Figure 1: Phylogenetic trees using the maximum likelihood of cysteine-protease (CP) sequences from *Tritrichomonas* spp., according to A) CP1, B) CP2, and C) CP4, using *Trichomonas vaginalis* as an outgroup.

Table 2. Statistics for the cysteine-protease (CP 1, CP2 and CP4) sequence alignments from *Tritrichomonas* spp., and the best nucleotide replacement model obtained.

Proteases	N	Variable sites	Preserved sites	Nucleotides	Replacement model	G-C percentage (%)
CP1	18	4	499	503	K2	21.77 – 14.79
CP2	33	28	641	669	K2+I	22.22 – 21.77
CP4	19	2	271	273	K2	20.17 – 24.55
Tándem	12	48	2235	2283	K2+G	22.60 – 21.24

G-C: Guanine-Cytosine

It is worth noting that the different *Tritrichomonas* spp. are grouped depending on the countries from which they were isolated, thus, being able to observe an apparent phylogenetic similarity in the pathogens of the same country, raising the possibility of evidencing potential entries of the disease and their probable relationships (Figure 1).

Finally, the tandem sequence focusing on giving a global overview of the relationships from the three cysteine-protease genes of *Tritrichomonas* spp. allowed improving the strength of the relationships and the separation of *Tritrichomonas* spp. isolated from cattle and pigs in a different group from the one comprised of those isolated from cats, bovines from Namibia, and primates (Figure 2).

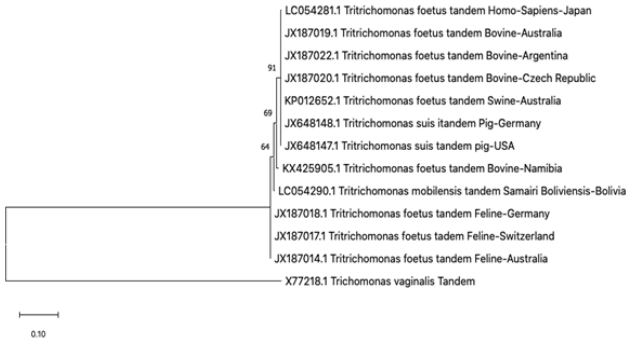

Figure 2: Phylogenetic tree using maximum likelihood and Bootstrap based on tandem sequences of cysteine-proteases CP1, CP2, and CP4 from *Tritrichomonas* spp., and *Trichomonas vaginalis* as an outgroup.

By carrying out the evolutionary divergence analysis of the 13 sequences built in tandem, the results obtained with the phylogenetic trees were able to be sustained; moreover, the similarity between the isolated sequences of cattle, pigs and humans that showed minimal divergence (0.0) was evidenced, and these, in turn, are distant from those of felines and non-human primates with a value of 0.023. The sequences of *Tritrichomonas mobilensis* from non-human primates, which despite being a different species from *T. foetus*, were evolutionarily closer to the sequences of feline *T. foetus* with values of 0.012, and more distant from those of human with 0.019. It can be pointed out that the sequences of feline *T. foetus* were closer to the sequences of *T. foetus* of bovines from Namibia with a value of 0.017 compared to the sequences of *T. foetus* from bovines, pigs and humans from other countries with a value of 0.023.

DISCUSSION

Tritrichomonas foetus has been considered a common parasite in cattle causing abortions, and in recent years, it has also been identified in cats producing chronic diarrhoea (9). In humans, some closely related parasites exclusive to the species, such as *Trichomonas vaginalis* (10) and *Trichomonas tenax* (11), as well as *Pentatrichomonas hominis* (7), have been found. These have also been isolated in domestic animals. Nonetheless, *Trichomonas foetus* has been detected as an etiologic agent of respiratory diseases and cholecystitis. On the other hand, in pigs, this specie, previously called *Tritrichomonas suis*, causes respiratory disease in human beings, suggesting the same etiological agent for various species (12).

Proteases are essential proteins for protozoan virulence and host-parasite interaction, but also in nutrient acquisition, induction of cellular apoptosis, adherence, metabolism and inflammation induction (13). For this reason, it is a good candidate for a phylogenetic evaluation. Furthermore, a study stated that using a multigenetic analysis of cysteine-proteases of different *Tritrichomonas* spp. genotypes isolated from felines, cattle, pigs and primates, there are conserved regions. This suggests that the genotypes of *T. foetus* from cattle originating from Namibia and felines in various countries of the world are associated with *T. mobiliensis* in primates (6).

When aligning 70 sequences of three different cysteine-proteases of *Tritrichomonas* spp. analyzed and reported in GenBank, the variant regions were 4, 28, and 2 for CP1, CP2, and CP4, respectively, evidencing a high variability of CP2. According to Šlapeta et al., this cysteine-protease is the most variable, so it is the most suitable to be used in phylogenetic analyses for these cases (6). The tandem sequence was constructed with the three proteases reported for each species of *Tritrichomonas* spp. isolated from different animals to obtain higher strength in the branches. The tandem sequences, being longer and showing all the variant sites, allowed observing more clearly the organization between tree clades. Moreover, it is important to note that *Trichomonas vaginalis*, despite being classified as *Trichomonadae*, is an evolutionarily different protozoan compared to *Tritrichomonas* spp. This situation has allowed it to behave as an outgroup in the phylogenetic analysis, as can be observed in the evolutionary divergence assessment (Table 3).

Table 2. Evolutionary divergence between the 13 cysteine-protease tandem sequences.

Sequence	1	2	3	4	5	6	7	8	9	10	11	12
1. LC054290.1 *T. mobilensis Saimiri bolivensis* - Bolivia												
2. JX648147.1 *T. suis* porcino USA	0.017											
3. JX648148.1 *T. suis* porcino Alemania	0.017	0.000										
4. KP012652.1 *T. foetus* porcino Australia	0.019	0.002	0.002									
5. LC054281.1 *T. foetus Homo sapiens* Japón	0.017	0.000	0.000	0.002								
6. JX187017.1 *T. foetus* felino Suiza	0.012	0.023	0.023	0.023	0.023							
7. JX187018.1 *T. foetus* felino Alemania	0.012	0.023	0.023	0.023	0.023	0.000						
8. JX187014.1 *T. foetus* felino Australia	0.012	0.023	0.023	0.023	0.023	0.000	0.000					
9. JX187020.1 *T. foetus* bovino Republica Checa	0.017	0.000	0.000	0.002	0.000	0.023	0.023	0.023				
10. KX425905.1 *T. foetus* bovino Namibia	0.013	0.015	0.015	0.017	0.015	0.017	0.017	0.017	0.015			
11. JX187019.1 *T. foetus* bovino Australia	0.017	0.000	0.000	0.002	0.000	0.023	0.023	0.023	0.000	0.015		
12. JX187022.1 *T. foetus* bovino Argentina	0.017	0.000	0.000	0.002	0.000	0.023	0.023	0.023	0.000	0.015	0.000	
13. X77218.1 *Trichomonas vaginalis* humano, Reino unido	1.036	1.057	1.057	1.059	1.057	1.020	1.020	1.020	1.057	1.040	1.057	1.057

By obtaining a better overview of the phylogenetic distribution of *Tritrichomonas* spp., and through a divergence analysis, a similarity was found between the *T. foetus* sequences of cattle and pigs. This result is similar to what Mueller et al (14) found in Australia where these authors compared cysteine-protease 1, 2, 4, and 9 from pigs and cattle that lived together, finding that the genotype present in the two species was the same. Furthermore, a prevalence of 65% of the bovine genotype has been reported in pigs even though this production is considered free of *T. foetus* in cattle for 30 years. This means that the parasite may be present in pigs of farms without producing disease in them, nor is a source of contagion to cattle (14).

Unlike this study where no similarity was found between the feline and porcine *T. foetus* genotypes using the cysteine-protease sequences, Doi et al (15) found that the genotypes identified in feline patients from some veterinary hospitals in Japan were the same as the genotypes reported in GenBank for pigs according to the TR/7 and TR/8 regions. Likewise, they found that both genotypes were different from bovine *T. foetus* (15). However, it is important to keep in mind that different genes were used for the analysis, and these may be more conserved and less related to invasion mechanisms. This result may reach different conclusions; nonetheless, it also increases the possibility that the barrier cross between species can occur for different genotypes of *Tritrichomonas* spp., a situation that must be studied in depth in other works with a higher number of genes.

In the phylogenetic tree, the sequences of bovine *T. foetus* are similar to each other, and there is very little genetic distance, except for the sequence of *T. foetus* isolated from bovines in Namibia that has a higher genetic closeness with the feline *T. foetus* that is located in the same clade and shows a minimal genetic divergence. It is noteworthy that the sequences of *T. foetus* from Namibian bovines and those from cats are sequences closer to *T. mobiliensis* compared to the sequences of *T. foetus* from other cattle, pigs or humans. Thus, the question about the accurate classification of these groups is

raised. It is important to note that the similarity between *T. foetus* from Namibian bovines and *T. mobiliensis* (6) has been previously reported and *T. suis* has been proposed as a genotype of *T. foetus* (12). This, in turn, raises the need to review the classification of some *Tritrichomonas* members. Nevertheless, this also suggests that at least two different genotypes of *T. foetus* can infect cattle and that each genotype is associated with different species, implying plasticity of the pathogen to cross between species. However, research using information from complete isolated genomes from different hosts is still lacking to clarify the situation.

A slight variation was evident between the sequences of feline *T. foetus*. Šlapeta et al. agree in affirming that the genotypes of bovine and feline *T. foetus* are different after comparing the TR/7 and TR/8 regions (16). Nonetheless, Morin et al (17) found a close similarity between the transcriptome when comparing both types of *T. foetus* in a study that aimed at identifying therapeutic targets for the treatment of this parasite, considering its adaptation to different species and the transcriptome changes due to this adaptation. According to these results, the authors conclude that this parasite has a recent evolutionary adaptation to the host (17). It is essential to highlight that studies such as the one of Morin, open the possibility of finding specific therapeutic targets given the difficulty of treatment in felines in which *T. foetus* shows resistance to most antiparasitic drugs except for ronidazole, highly toxic in treated animals (17). Similar findings were obtained by Reinmann et al (18) who compared ITS 2 sequences and the elongation factor to 1 as a semi-conserved sequence, finding that the sequences of both loci are phylogenetically very close (18). These similarities open the door to future studies considering that in many bovine production systems where concentrate feed is stored, cats are found controlling rodents that visit these sites, making them a source of contagion for beef cattle.

Conversely, in Mantes, France, in 2006, a case of pneumocystis pneumonia was reported in a 54-year-old woman caused by *T. foetus*. The diagnosis of the etiological agent was carried out through PCR of the ITS 1 region, and its sequence was compared to those found in databases. The sequences showed homology of 99.2% concerning the sequences of *T. foetus*, *T. suis,* and *T. mobilensis* found in cattle, pigs and squirrels (19). Another case reported in humans was made in Japan, where *T. foetus* was isolated from the bile duct from a patient with cholecystitis. After isolation, genotyping was performed at CP 1, 2, 4, and 9 and was compared with the *T. foetus* sequences of cattle and pigs, showing to be the same genotypes (8).

All these results warn us about the importance of *T. foetus,* not only as a cause of disease in cats and animals for slaughter but also due to a possible zoonosis, especially if all potential transmitters towards man are considered. Furthermore, also because there are no systematic studies conducted in humans who have frequent contact with susceptible species. Although some studies show that similar genotypes of *T. foetus* are found in different species without causing disease (14), other reports show patients such as pigs and humans with respiratory diseases, cats with intestinal problems, and cattle with different reproductive problems. In this sense, future research is necessary to compare the genotypes of this parasite in the species mentioned above, considering that it is common for both bovine and porcine production systems to have cats in them, in addition to having close human contact with these animals (20).

Nonetheless, having only one known report in Latin America of *T. foetus* in cats (1), it is essential to carry out prevalence studies in this region considering the high prevalence in developed countries such as Germany, Australia, and Switzerland (6).

Finally, when carrying out the analysis of the trees by geographic distribution, a similarity of pathogens was observed according to the countries where the samples were taken. This means that these sequences can be useful to clarify the origin of these parasites, as well as possible cross-contamination between animal species, so they should be considered in future studies in Latin America to identify the possible origin of the pathogen.

In conclusion, in the current study, the phylogenetic distances of the different cysteine-protease sequences analyzed were estimated, suggesting closeness between the *Tritrichomonas foetus* isolated in felines and bovines from Namibia, and *Tritrichomonas mobilensis* in non-human primates, but slightly more distant from the group formed by *T. foetus* from cattle, and *T. suis* from pigs and a human sequence. This

closeness and the fact of finding sequences of bovine *T. foetus* in the two groups suggest the possibility of crossing the barrier between species. However, *in vivo* molecular studies with different populations of related animals and humans are required to confirm this conclusion.

Conflict of interests

The authors declare that there are no conflicts of interest in this study.

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