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Tritrichomonas – Systematics of an enigmatic genus

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ABSTRACT

Tritrichomonas spp. are parasitic protozoans that proliferate on mucus membranes of the urogenital, gastro-intestinal or nasal tract. For instance, *Tritrichomonas foetus* is an important cause of reproductive failure in cattle. Some years ago, *T. foetus* was also identified as a causative agent of diarrhoea in cats. Previous studies on the morphological, physiological and molecular levels have raised doubts as to the phylogenetic relationship among some *Tritrichomonas* species, particularly in relation to *T. foetus, Tritrichomonas suis,* and *Tritrichomonas mobilensis*. With the advent of molecular genetic tools, it has become clear that these three tritrichomonad species are closely related or may even represent the same species. Indeed, since recently, *T. suis* and *T. foetus* are generally considered as one species, with *T. mobilensis* being a closely related sister taxon. To date, molecular studies have not yet been able to resolve the taxonomic (specific) status of *T. foetus* from cattle and cats. In the future, novel genomic approaches, particularly those involving next generation sequencing are poised to resolve the taxonomy of *Tritrichomonas* spp. Here, we review the literature on the current state of knowledge of the taxonomy of *T. foetus*, *T. suis*, and *T. mobilensis* with special reference to the relationship between *T. foetus* from cattle and cats.

1. Introduction

Members of the family Trichomonadidae (Phylum Parabasalia) are protozoans characterized by a parabasal body, 3 to 5 anterior flagella, an undulating membrane which forms a posterior flagellum and an axostyl. Trichomonads are early branching protists [1], i.e. they are basal from an evolutionary perspective. Indeed, a recent study suggested that dinosaurs were affected by trichomonads [2]. Traditionally, the members of this family were identified and differentiated based on morphological features (number of anterior flagella; *Tritrichomonas* spp., *Tetratrichomonas* or *Trichomonas* spp., *Pentatrichomonas* spp.) and host origin. Although many members of the family have low pathogenicity or are regarded as harmless commensals of the intestine, the nasal, or the oral cavity, some trichomonads cause very important diseases of both, human and veterinary medical relevance. *Trichomonas vaginalis*, for instance, causes the most important sexually transmitted, non-viral disease of humans worldwide [3]. *Tritrichomonas foetus* is a venereally transmitted pathogen that affects cattle and causes important economic losses to the livestock industry [4]. *Trichomonas gallinae* affects birds and can cause severe, or even fatal, disease of the proximal digestive tract. Recently, it has been identified as causative agent of an emerging disease of European finches [5].

The first available full genome of trichomonads is that of *T. vaginalis* [6]: It is ~160 Mb in size and contains a substantial amount of repetitive DNA and heterogeneity. The region comprising the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) and the intervening 5.8S rRNA gene has been used for species identification of trichomonads [7]. However, due to limited sequence variation in this DNA region among some trichomonads, doubts have arisen regarding their specific status. For instance, *T. foetus* from cattle has been found to be identical in sequence to *Tritrichomonas suis* [7–9], a commensal in the nasal cavity and intestine of pigs, and also very similar to *Tritrichomonas mobilensis* [7,9], a tritrichomonad found in the intestinal tract of squirrel monkeys and possibly other mammals [10,11]. Recently, another very closely related, or identical, species to *T. foetus* has been identified in cats [12]. Already in 2002 a study by Tachezy et al. [9] proposed that *T. foetus* and *T. suis* were synonyms, and an opinion supporting this was published in 2005 by Lun et al. [13]. Based on these works, GenBank now refers to *T. foetus* as *T. suis*. With the occurrence of feline *T. foetus*, new discussions have arisen as to whether feline and bovine *T. foetus* represent the same or distinct species. Here, we review the current state of knowledge of the taxonomy of *T. foetus*, *T. suis* and *T. mobilensis*, with a special emphasis on *T. foetus* from cattle and cats, and the new molecular tools that are used for specific or genotypic identification.

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2. Clinical relevance and epidemiology of *Tritrichomonas* spp.

From a clinical view point, the most important species of tritrichomonad is *T. foetus*. This parasite is transmitted venereally and causes a disease affecting cattle, which is responsible for substantial economic losses, particularly in beef herds [4]. Although bulls remain asymptomatic carriers, female animals suffer from vaginitis, endometritis and infertility, but are able to clear the infection within some months [4]. In many countries, regulations have been implemented to control this disease [14], and artificial insemination (AI), together with the rigorous diagnostic monitoring of the bulls used for AI, has succeeded in eradicating tritrichomonosis from cattle in many European countries [15]. The parasite is still prevalent in some regions, particularly in the Americas, South Africa, Australia, and East European countries, where cattle are farmed extensively and are allowed to mate naturally [16].

Approximately a decade ago, parasites identified first as trichomonads [17] and later as *T. foetus* [12] were shown to cause chronic large-bowel diarrhoea in domestic cats in the USA. Subsequently, numerous reports from the USA, Europe and Australasia [17–27] have revealed that the infection in cats is widespread and may be considered as an emerging disease. In cats with a history of diarrhoea, prevalence can be >25% [18,23,26]. Feline tritrichomonosis first seemed to be a problem of pedigree cats [17–19,22,23], but recent publications from the USA and Italy have indicated that the infection is also widespread in cross-bred domestic cats [26,28]; dense housing has been identified as a risk factor for infection [18].

T. foetus is not generally recognized as a zoonotic agent, but a fatal peritonitis and a fatal meningoencephalitis caused by *T. foetus* in two immunocompromised patients have been described [29,30]. In an AIDS patient, *T. foetus* was found to be involved as co-infecting agent in a *pneumocystis* pneumonia [31].

T. suis is a commensal found in the nasal cavity and the intestine of pigs. As it is non-pathogenic in this species of host [32], little is known about the parasite's prevalence. For example, one study found a high prevalence of 68–90% in domestic pigs [33], and another study reported a prevalence of 25% in wild boar in Iran [34]. Very little information is available also for *T. mobilensis.* There are no studies of wild mammals, but in laboratory colonies of squirrel monkeys, prevalence appears to be up to 100% [35]. Although *T. mobilensis* infection does not appear to cause pathological changes in these hosts, this trichomonad has been shown to be associated with liquefied caecal content in tree shrews [11] and gastritis in macaques co-infected with simian immunodeficiency virus [36]. In the following sections, we focus on the above-mentioned tritrichomonads, because there is very little molecular information on other tritrichomonads.

3. The debate regarding taxonomic status

The classification of *T. suis* and *T. foetus* as two separate species, based on host origin and infection site, has been under scrutiny. Studies of *T. suis* [37,38] and *T. foetus* [39,40] have revealed close morphological similarity. Other studies, directly comparing the two tritrichomonads, have not revealed any specific differences, although some variations in morphology (such as plump or slender forms) have been observed [9,41]. Plump or slender forms have been detected for both *T. foetus* and *T. suis*, indicating that these characters are not specific [9]. Also the karyotype has been shown to be identical for *T. foetus* and *T. suis* [42], and no differences could be observed between the two taxa on an ultrastructural level [43]. Furthermore, studies of physiological parameters [43–47] as well as antigenic properties [48–52] have not revealed any distinct differences between *T. foetus* and *T. suis*.

Importantly, cross-infection experiments between pigs and cattle with *T. foetus* and with *T. suis* have been successful [53–55]. In cattle, *T. suis* infection led to vaginal catarrh and was transmissible by coitus [53]. Conversely, Cobo et al. [56] could not establish an infection with *T. suis* in heifers, although a measurable humoural immune response against the parasite was detected.

The occurrence of *T. foetus* in cats has stimulated some investigations of its identity. As *T. foetus* in cats was only discovered in the late 1990s, most studies used molecular approaches to compare this species with other trichomonads. In addition, one experiment indicated successful artificial infection of heifers with *T. foetus* from cats [57], resulting in vaginal discharge and histological lesions. However, these signs and changes were similar, but not identical, to the control infection with *T. foetus* from cattle. Infection of cats with bovine *T. foetus* resulted in less successful intestinal colonisation as compared with the control isolate from cats [58]. Morphologically, *T. foetus* from cats and cattle seemed to be identical, with the occurrence of both plump and slender forms (Fig. 1).

4. Genotyping of *Tritrichomonas* spp.

The different molecular tools that have been used to characterise *Tritrichomonas* spp. are summarised in Table 1. Variable length repeats (VLR) were first used by Riley et al. [59] as genetic markers for characterisation of *T. vaginalis*. Subsequently, the same authors applied the same marker to the characterisation of *T. foetus* from cattle. In particular, gel-electrophoretic banding patterns, containing several bands of differential sizes and only one common 220 bp band, indicated a considerable level of intra-species variation between different bovine isolates of *T. foetus* [60]. Tachezy et al. [9] used identical primers (TR7/TR8) for VLR-PCR to confirm the identity of *T. suis* and *T. foetus* from cattle. These authors found that *T. foetus* from cattle, *T. suis* and *T. mobilensis* were indistinguishable in that they exhibited the same ladder of amplicons (of 110, 210, 320, and 502 bp). Cloning and sequencing of the 502 bp amplicon revealed complete sequence identity between *T. suis* and bovine *T. foetus*, while *T. mobilensis* differed by 5 nucleotides in this sequence. Recently, the same primer pair has been used for a PCR-based study to compare bovine and feline *T. foetus* isolates [61]. Here, 320 bp and 210 bp regions were amplified consistently, along with some other bands that differed between isolates. The sequencing of the 320 bp amplicon revealed 11 consistent differences between feline and bovine *T. foetus* isolates.

Other approaches applied to *T. foetus* from cattle, *T. suis* and *T. mobilensis* include restriction fragment length polymorphism

Fig. 1. Scanning electron micrograph of bovine *Tritrichomonas foetus*. The trichomonads are grown on a monolayer of bovine vaginal epithelial cells. Both plump and slender forms can be observed. This image was kindly provided by A. Hemphill.

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Table 1
Summary of the different methods that have been used to genetically characterise tritrichomonads.

Method	Primers/length of amplification product	Results	References	Notes
Variable length repeats (VLR)	TR7/TR8	Intra-species variation in bovine *T. foetus* isolates	[60]	Low repeatability between laboratories
	TR7/TR8	Bovine *T. foetus*, *T. suis* and *T. mobiliensis* undistinguishable	[9]	
	TR7/TR8	Intra-species variations in bovine and feline *T. foetus* isolates	[61]	
Sequencing of VLR	502 bp	Bovine *T. foetus* and *T. suis* identical; *T. mobiliensis* differed in 5 SNPs	[9]	Low repeatability between laboratories
	320 bp	11 SNPs difference between feline and bovine *T. foetus* isolates	[61]	
RFLP	None	*T. suis* and bovine *T. foetus* identical, *T. mobilensis* similar but distinguishable	[9]	Intra-species variation in *T. foetus* and *T. suis* isolates
RAPD	TAP5/TAP6	Intra-species variation in bovine *T. foetus* isolates	[60]	
	20–29 random primers	Bovine *T. foetus* and *T. suis* identical, *T. mobilensis* distinguishable	[8,9]	
ITS-1, 5.8S rRNA, ITS-2	ca. 300 bp	Bovine *T. foetus* and *T. suis* identical; *T. mobilensis:* 1 SNP difference; feline *T. foetus:* 1 SNP difference	[7,61,68]	High repeatability in various studies
EF-1α	ca. 800 bp	5 SNPs difference between feline and bovine *T. foetus*	[68]	PCR able to discriminate between feline and bovine isolates; no sequencing needed
CP8	Ca. 650 bp	2 SNPs difference between feline and bovine *T. foetus*	[69]	
RPb1	ca. 5000 bp	Phylogenetic tree of parabasalia	[72]	Not yet used for comparison of tritrichomonads
Microsatellites	Various primers	Intra-species variation in *T. vaginalis* isolates.	[73,74]	Not yet used for tritrichomonads

(RFLP) analysis [9], and randomly amplified polymorphic DNA analysis (RAPD) [8,9,60]. Both approaches were selected for analyses of genomic DNA from different tritrichomonad isolates. In these analyses, *T. mobilensis* exhibited a banding pattern that was distinct from those of *T. foetus* and *T. suis* [8,9]. Conversely, respective RFLP and RAPD banding patterns were not suited for consistent discrimination between *T. foetus* and *T. suis* [8,9]. However, variable features within the complex banding patterns indicated variability within *T. foetus* and *T. suis* [9,60].

The considerable intra-taxon variation and the low reproducibility of RFLP, RAPD, and assessment of variable length repeats made it necessary to identify other genomic markers that could be used for an unambiguous characterization of different tritrichomonads. Therefore, Felleisen et al. [7] used a rDNA region, comprising the 5.8S rRNA gene as well as ITS-1 and ITS-2 for the genetic characterisation of tritrichomonads. The development of the PCR was based on sequence information on the small subunit RNA and the 5.8S rRNA gene of *T. foetus* [62]. This combined marker has been most widely used for the identification of trichomonads [23,61,63–68]. For *T. foetus* from cattle and *T. suis*, complete identity could be shown in this region [7,65,68]. For *T. mobilensis*, only a single nucleotide polymorphism (SNP) was detected in the ITS-2 region, with complete identity in the ITS-1 and the 5.8S rRNA gene [7]. Therefore, in these studies, no genetic difference was detected between bovine *T. foetus* and *T. suis*, which led to the synonomy of *T. foetus* [70] and *T. suis* [13,71]. Because *T. suis* historically is the older name, the GenBank database now refers to all submitted *T. foetus* sequences as *T. suis*. The ITS-2 region has been established as discriminating region between *T. foetus* isolated from cats and from cattle with one SNP differing between the two isolates [61,68]. Also in the case of the fatal human peritonitis, the ITS-2 locus was used to demonstrate 100% identity of the trichomonads with *T. foetus* from cattle [29].

The wide occurrence of feline *T. foetus* infection in countries free from bovine *T. foetus* has encouraged molecular epidemiologists to hunt for more genomic markers. The successful whole genome sequencing of *T. vaginalis* [6] has boosted the development of such approaches. Very recently, a novel gene marker has been identified in our lab that allows the discrimination of feline from bovine *T. foetus* isolates. Here, the partial coding sequence of the elongation factor-1α (EF-1α) gene was used by Reinmann et al. [68] to compare bovine and feline *T. foetus* isolates as well as *T. suis*, *T. mobilensis* and other trichomonads. This study revealed 5 nucleotide polymorphisms to distinguish bovine from feline *T. foetus* isolates. In the same study, the use of a forward primer matching the bovine-type variable region of the EF-1α gene and a reverse primer covering a conserved EF-1α region allowed the specific PCR-based detection of bovine *T. foetus* isolates, but not feline isolates [68]. As *T. suis* and *T. mobilensis* were identical to the feline sequences in the forward variable region, the primer combination using the bovine-type forward primer did not amplify these sequences [68]. Compared with ITS-2 (Fig. 2A), the EF-1α sequence (Fig. 2B) exhibited higher variability and thus allowed a better phylogenetic resolution between *T. suis* and *T. mobilensis* as well as between bovine and feline *T. foetus* isolates. Most likely due to the multi-copy nature of the EF-1α gene, the primer combination including the feline-type variable part of EF-1α (forward primer) and the conserved reverse primer, was not able to distinguish between feline and bovine isolates.

A second novel gene marker has been identified by Sun et al. [69]. This study revealed two SNPs unambiguously distinguishing feline from bovine *T. foetus* sequences in a stretch of 663 bp of the coding region of the cysteine proteinase 8 (CP8) gene. One of the SNPs led to an amino acid substitution. As CP8 is probably involved in the pathogenic effects of *T. foetus*, differences in the coding region resulting in a different amino acid sequence might potentially help to understand biological differences between the isolates [69].

An improved approach for PCR-based genotyping of trichomonads might include the use of single copy genes as targets for specific amplification. Here, a promising candidate is the gene encoding the largest subunit of the RNA polymerase II (Rpb1) [72]. This gene is about 5 kb in size and is free from introns. It was successfully applied to establish a phylogeny for parasitic and free-living parabasalia [72]. However, this study only used one bovine *T. foetus* isolate as a representative of the tritrichomonads. In future, similar studies comparing bovine and feline *T. foetus,* as well as *T. suis* and *T. mobilensis,* should be performed in order to reconstruct the phylogeny of tritrichomonads.

Based on the public availability of the whole genome sequence of *T. vaginalis* (http://trichdb.org/trichdb/), also microsatellite analyses became feasible for genetic differentiation between or among trichomonad isolates. Indeed, two laboratories identified candidate sequences for a microsatellite-based genotyping of *T. vaginalis* [73,74]. Both investigations found polymorphisms in

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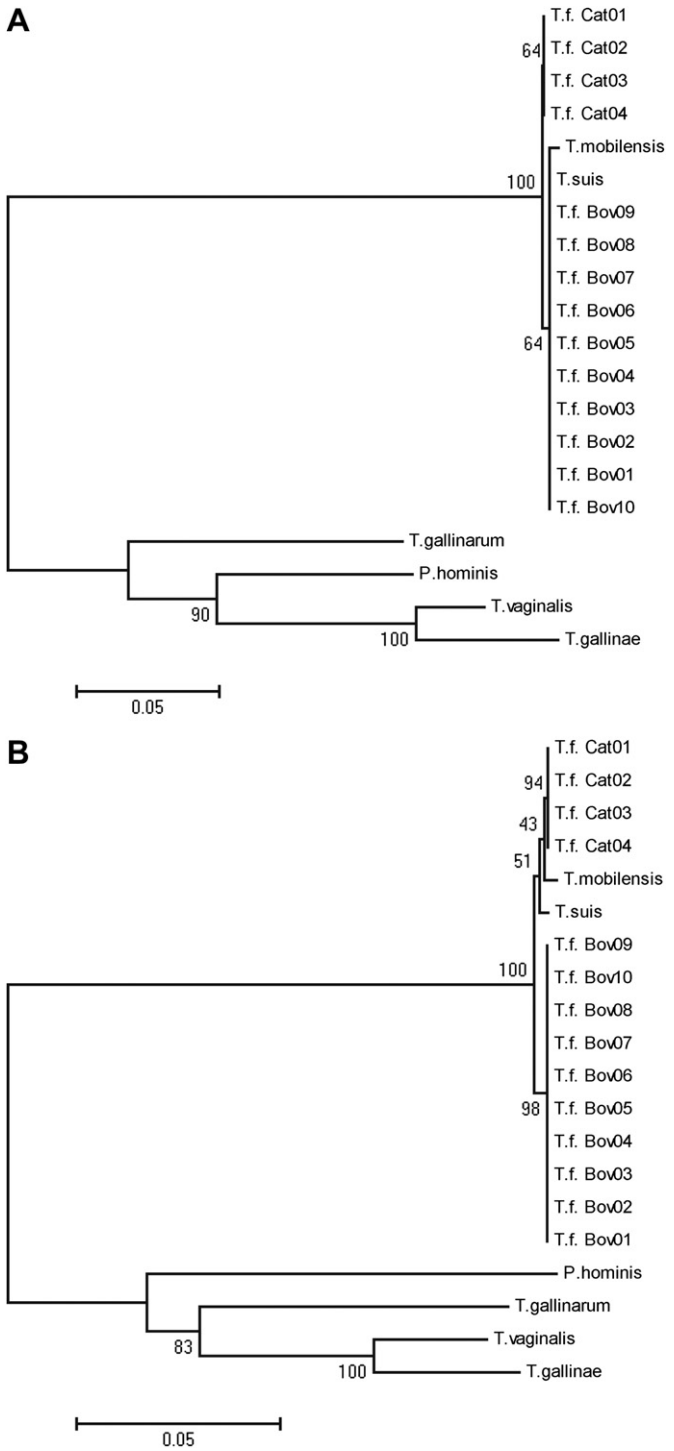

Fig. 2. Phylogenetic relationship between ITS-2 (A) and partial EF-1α (B) sequences from feline and bovine *T. foetus* isolates, *T. mobilensis, T. suis, T. vaginalis, T. gallinae, T. gallinarum* and *P. hominis*. The phylogenetic trees were drawn using the neighbour-joining method and the Kimura two parameter model of correction (MEGA software v. 5.05, http://www.megasoftware.net). Branch lengths are proportional to sequence divergence and relate to the scale bar shown (bottom left). The scale represents nucleotide substitution per position. The figure has been adapted from the supplementary material to Reinmann et al. [68].

different laboratory strains of *T. vaginalis*, highlighting the complex nature of these protozoan parasites. The study of Conrad et al. [73] also identified 21 single copy genes exhibiting multiple SNPs among different isolates of *T. vaginalis*.

5. Conclusions

It is evident that the currently available information gained from morphological, physiological and transmission studies is not yet adequate to identify and delineate tritrichomonad species. Thus, molecular investigations have gained increased importance. Despite extensive efforts, to date, no genetic marker allows the unequivocal identification and differentiation of tritrichomonad species. In addition, the phylogenetic position of feline *T. foetus* is still unresolved. Recent studies have detected small differences between bovine and feline isolates in some regions of the genome. However, whether these SNPs justify the division of feline and bovine *T. foetus* into two genotypes or species remains unclear. Furthermore, subtle genomic differences between *T. foetus* and *T. suis* have been shown [68] that might indicate that these two species are not synonyms after all. Therefore, whole genome sequencing of feline and bovine *T. foetus*, as well as of other tritrichomonads, is highly desirable. Novel molecular approaches, which are made possible by the availability of the complete genome sequence, such as microsatellite studies or the analysis of single copy genes, should provide an increased resolution of the systematics of the complex family of the Tritrichomonadidae and might also provide the basis for epidemiological and ecological studies as well as improved diagnostic tools.

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