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Fine structure and isozymic characterization of trichomonadid protozoa

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Abstract *Tritrichomonas suis* and *T. foetus* are characterized herein at the ultrastructural and biochemical levels. Microcinematography and measurements, scanning and transmission electron microscopy, cytochemistry for carbohydrate detection (Thiéry technique), and isozyme electrophoresis analysis were performed. In all, 11 different strains from 5 species of parasites were studied (*T. foetus*, *T. suis*, *Trichomonas gallinae*, *T. vaginalis*, and *Monocercomonas* sp.). A total of 11 enzymes were scored. Fine-structure study using scanning and transmission electron microscopy demonstrated that *T. suis* and *T. foetus* are identical morphologically. The high degree of isozymatic similarity noted between *T. suis* and *T. foetus* is consistent with the hypothesis that they may be different strains of the same species.

Introduction

Tritrichomonas suis (Gruby and Delafond 1843) is a trichomonadid protozoan living in the nasal cavity and digestive tract (principally the stomach, small intestine, cecum, and colon) of swine (Levine 1973). *T. foetus* (Riedmuller 1928) is a similar species of the genus that parasites the urogenital tract of cattle. These two species are very similar in their physiology (Doran 1957; Lindblom 1961) and share many antigens (Kerr 1958; Robertson 1960; De Carli and Guerrero, 1975, 1976, 1977). Furthermore, cross-infection studies did not find any significant host specificity differences between *T. suis* and *T. foetus* (Fitzgerald et al. 1958), which led their specific status to be questioned (Robertson 1960; Honigberg 1978). Finally, specimens attributed to these two species on the basis of the host from which they were isolated did not show any clear morphological differences under the light microscope (Hibler et al. 1960). A recent study compared some properties of sialic-acid-binding systems in *T. suis* and *T. foetus* using hemagglutination of normal and enzyme-treated red blood cells and its inhibition, adherence to porcine cecal mucus, and kinetic properties of neuraminidase (Pakandl and Grubhoffer 1994). At present it is not quite clear whether *T. suis* and *T. foetus* are separate species. It thus appears open to doubt whether these two putative species should be kept as separate taxa or whether they represent just ecotypes of one single species. There has been no ultrastructure study on *T. suis*, although some authors claim that *T. foetus* and *T. suis* could appear identical under electron microscopy.

Herein we report on the ultrastructure and the cytochemistry of *T. suis* and compare them with those of *T. foetus*. We also analyzed the isozymes of several strains from both nominal taxa to estimate their genetic similarity as compared with that of other trichomonads. Both approaches corroborate the hypothesis that *T. suis* is a senior synonym of *T. foetus* or that *T. foetus* is a host-defined strain of *T. suis*.

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Materials and methods

The following cultures were used in the present study: *Tritrichomonas foetus* [five strains: strain K from the American Type

Culture Collection (ATCC) and the others (strains 5022, PAL, RJ, and V1) isolated by one of us (G.D.) from the urogenital tract of bulls from the state of Rio Grande do Sul, Brazil]; *T. suis* strains 169 and 167 from the ATCC and strain TSM isolated by one of us (G.D.) from a pig in the state of Rio Grande do Sul, Brazil; three strains of *Trichomonas gallinae* (strains TG14, TG9, and TG11); and three strains of *T. vaginalis*, strains 30238 and 30236 from the ATCC and the JT strain isolated from a patient in the University Hospital, Rio de Janeiro, Brazil. *Monocercomonas* sp. from *Natrix sipedon* was obtained from Dr. J. Kulda (Charles University, Prague). All cells were maintained in Diamond's TYM medium (Diamond 1957) and were cultivated for 24 h at 36.5 °C, except for *Monocercomonas*, which was cultivated at 28 °C.

Microcinematography and measurements

Living cells were observed and filmed in a Zeiss Axioplan light microscope coupled with a high-resolution video-camera (Pasecon-Pal-G system/Grundig electronic or NTSC-optronics camera) and the digital image-processing system IBAS (Kontron-Zeiss), attached to the same microscope. The video images were subjected to a process of analog contrast enhancement and digitized, and then a background defocused image was subtracted. Afterward, a special procedure to enhance contours was used in each frame. Frozen images were transferred to the software of a Zeiss 912 electron microscope, and length and width measurements were taken of 100 cells.

Scanning electron microscopy

The cells were collected by centrifugation at 1,500 *g* for 5 min and fixed with 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer at pH 7.2 for 2 h. After being rinsed in phosphate buffered saline (PBS) parasites were left for 10 min to adhere to poly-L-lysine-coated glass coverslips. They were then postfixed in 1% OsO_4, dehydrated in ethanol, and then critical-point-dried using CO_2. The coverslips were coated with gold and observed in a Zeiss EM 962 scanning electron microscope.

Transmission electron microscopy

The parasites were fixed for 2 h at room temperature in a solution containing 2.5% glutaraldehyde, 4% paraformaldehyde, and 5 m*M* $CaCl_2$ in 0.1 *M* cacodylate buffer (pH 7.2). After being rinsed in PBS, the cultures were postfixed with 1% OsO_4 in 0.1 *M* cacodylate buffer plus 0.8% potassium ferricyanide. They were then dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss EM 900 electron microscope. Thiéry technique (1967) was performed as previously described (Benchimol et al., 1996). After Thiéry technique the same carbohydrate distribution was observed in *T. suis* and *T. foetus* (not shown).

Isoenzyme electrophoresis

Cultures of the 11 different cells studied were homogenized by sonication in a W-380 Ultrasonic Processor for 30 s in a hypotonic solution of 1 m*M* dithiothreitol, 1 m*M* aminocaproic acid, and 1 m*M* ethylenediaminetetraaceticacid (EDTA). The lysates were frozen in liquid nitrogen and analyzed electrophoretically the following day.

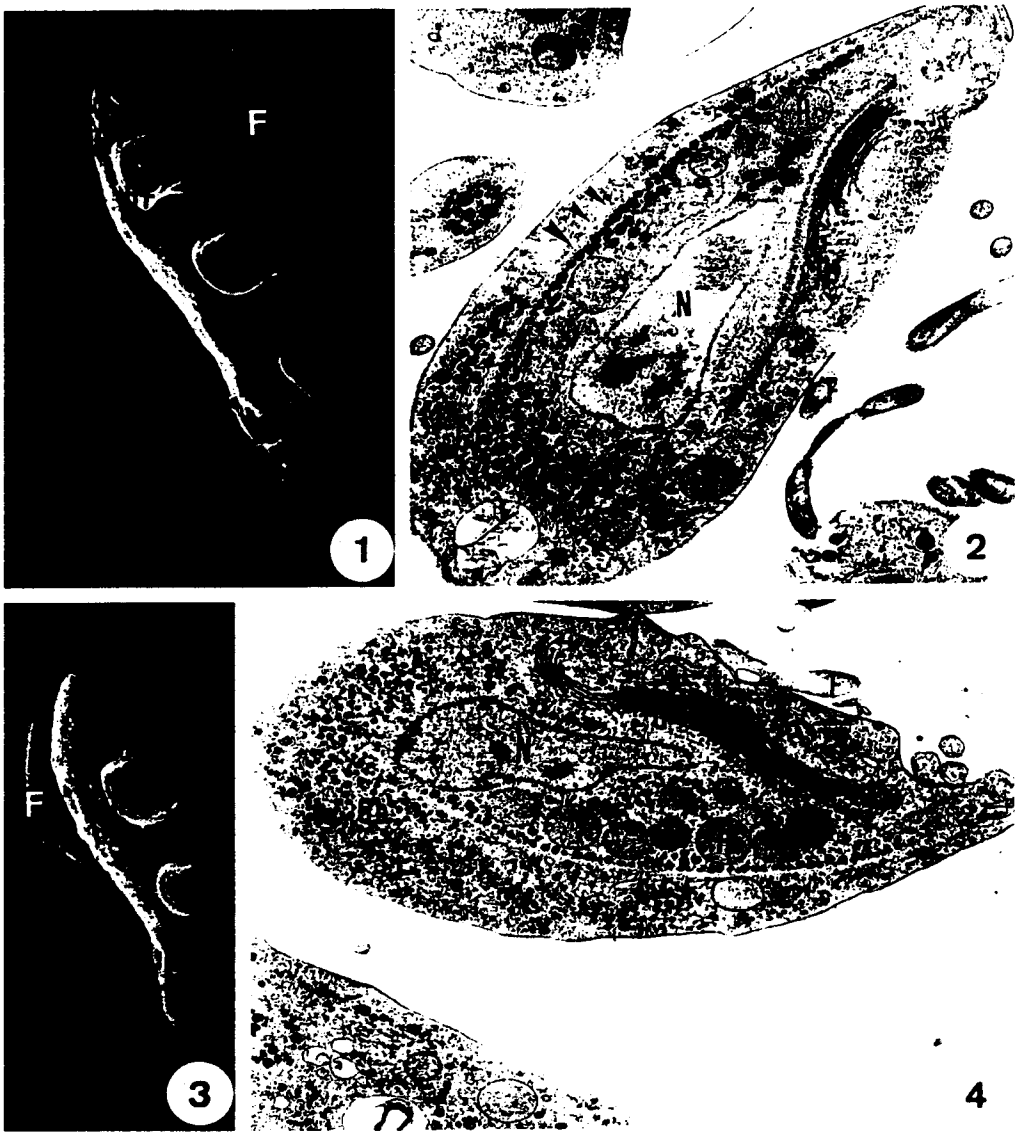
The isozyme patterns of the species were obtained by 13% horizontal starch-gel electrophoresis as previously described (Motta et al. 1991). Staining solutions and enzyme nomenclature follow those described by Hillis and Moritz (1990). A total of 16 enzymes were assayed in 3 buffer systems [discontinuous TRIS-citrate/borate (Poulik 1957), TRIS-EDTA-maleate, pH 7.4 (Miles et al. 1980); and TRIS-citrate, pH 8.0 (Ward and Beardmore 1978)], of which 11 (phosphoglucomutase, PGM, E.C.2.7.5.1; acid phosphatases, ACP, E.C. 3.1.3.2; pro-phe peptidase, PEP, E.C.3.4.11.1; hexokinase, HK, E.C. 2.7.1.1; phosphogluconate dehydrogenase, PGD, E.C. 1.1.1.44; phosphoglucose isomerase, PGI, E.C. 5.3.1.9; alpha-esterases, EST, E.C. 3.1.1.x; malic dehydrogenase, MDH, E.C. 1.1.1.37; catalase, CAT, E.C. 1.11.1.6; glucose 6-phosphate dehydrogenase, GPD, E.C. 1.1.1.49; and malic enzyme, ME, E.C.1.1.1.40) could be reliably scored. For the facilitatism of interpretation, all samples were run twice on the same gel. Complete replicate experiments (including culture growth and eletrophoresis) were also done with all strains to confirm the observed banding patterns. After being stained, the gels were scored and preserved in 20% glycerol in 7% (v/v) acetic acid.

The electrophoretic data were interpreted conservatively by elimination from the analysis of bands that might have been the result of heterohybridization (both within heterozygotes and between loci) in the multimeric enzymes. Electromorphs were then used to build a binary matrix (presence/absence) that was analyzed using the implicit enumeration option of the DOLLOP (minimizing reversals) and MIX programs of the computer package PHYLIP (Felsenstein 1985), with *Monocercomonas* sp. serving as an outgroup.

Results

In the present study, complementary techniques were used to analyze the structural organization of *Tritrichomonas suis* as well as its relationship with *T. foetus*. Examination of living cells by differential light microscopy and by microcinematography or of previously fixed cells by scanning electron microscopy (SEM) showed the same overall size, shape, and number, and strain 169 measured 16.5 (12.7–22.2) X 6.7 (4.6–9.6) μm. The cells of strain 167 measured 14.9 (10.8–20.0) X 6.8 (4.4–8.9) μm. We also measured *T. foetus* using the same methodology (living cells, distribution of flagella for *T. suis* versus *T. foetus*, living specimens of strain frozen images). We found the K strain to measure 16.1 (2.9–9.7) X 6.3 (2.9–9.7) μm. Typically, the flagellate's body was ovoid in nondividing cells and presented a sharp tip (Figs. 1, 3). Small pits were seen over the cell surface by SEM (Figs. 1, 3).

Three anterior flagella and one recurrent flagellum were observed (Figs. 1–4). They emerged from the periflagellar canal (Figs. 1, 3). The area of emergence of the recurrent flagellum was separated from the three anterior flagella by a narrow strip of cytoplasm (Figs. 1, 3). One of the three anterior flagella was always in different stages of movement (Figs. 1, 3). Examination of thin sections showed that *T. suis* and *T. foetus* had the same ultrastructure: the size, shape, and anterior position of the nuclei were similar and the hydrogenosomes were mainly concentrated in the region close to the axostyle and costae for both samples (Figs. 2, 4). The hydrogenosomes also presented similar fine-structural aspects such as a double membrane and peripheral compartments with calcium deposits, which were also surrounded by a double membrane. Furthermore, the position and general aspect of the Golgi complex, the glycogen distribution (Figs. 2, 4), the mastigont system with its basal bodies and accessory filaments, and the emergence of the costae and presence of a comb in *T. suis* were identical to those previously described for *T. foetus*.



Figs. 1–4 General aspect of *Tritrichomonas foetus* (**Figs. 1, 2**) and *T. suis* (**Figs. 3, 4**) as seen by SEM (**Figs. 1, 3**) and transmission electron microscopy (**Figs. 2, 4**). The protozoan body is ovoid and has a sharp tip. It presents three anterior flagella (*F*) emerging from the periflagellar canal. The recurrent flagellum (*RF*) emerges from a different exit close to the periflagellar canal and runs across the cell body as an undulating membrane (**Figs. 1, 3**). The nucleus (*N*) occupies the central to anterior region of the cell and is not a round structure. The hydrogenosomes (*H*) are distributed close to the axostyle (*arrowheads*) and to glycogen granules (*GL*). The Golgi complex (*G*) is a prominent structure. **Fig. 1** X 8,000. **Figs. 2, 4** X 16,000. **Fig. 3**, X 6,000

In both *T. suis* and *T. foetus* the parabasal apparatus was a 3- to 4-μm-long structure situated dorsally and to the right of the nucleus (Figs. 2, 4). It varied in size and shape, depending on the plane of sectioning. The proximal part of the undulating membrane was attached to the recurrent flagellum by thin filamentous bridges (data not shown). Membranous profiles were seen in the proximal marginal lamella. The microtubules of the distal part of the undulanting membrane as well as their protofilaments were clearly visible (data not shown).

T. foetus and *T. suis* differed at only 2 of the 42 electromorphs analyzsed (Table. 1). The five most parsimonious trees were produced with both the MIX parsimony and the DOLLO parsimony programs. The trees differed only in the relative positions of the different strains within each species (a strict consensus tree is shown in Fig. 5).

Catalase enzymes (two different electromorphs) were observed in *T. foetus* and *T. suis*, although they were not found in *T. augusta*, *Trichomonas vaginalis*, or *T. gallinae*.

Discussion

The most important finding of this study is the level of morphological and genetic similarity between two putative species of *Tritrichomonas*. Previous studies using

Table 1 Allele distribution for the 11 enzymes studied in *Trichomonas suis*, *T. foetus*, *T. gallinae*, *T. vaginalis* and the outgroup *Monocercomonas* sp. *ACP* acid phosphatase, *CAT* catalase, *ES* alpha-esterase, *GPD* glucose 6-phosphate dehydrogenase, *HK* hexokinase, *MDH* malic dehydrogenase, *ME* malic enzyme, *PEP* pro-phe peptidase, *PGD* phosphogluconate dehydrogenase, *PGI* phosphoglucose isomerase, *PGM* phosphoglucomutase, ? no band developed on the gel for a given enzyme

Species	Strain	ACP	CAT	EST	GPD	HK	MDH	ME	PEP	PGD	PGI	PGM
T. vaginalis	30236	001100	001	0	10	000001	01	????	0010	0000010	100	0110
	30238	001100	001	0	10	000001	01	????	0010	0000010	100	0110
	JT	001100	001	0	10	000001	01	????	0010	0000010	100	0100
T. gallinae	TG9	110100	001	0	10	001000	10	1110	1100	1010000	011	0100
	TG11	000001	001	0	10	001100	10	1110	0100	1010000	011	0110
	TG14	110100	001	0	10	001100	10	1100	1100	0010000	???	????
T. foetus	K	011000	100	0	10	110000	01	0010	0100	0010100	010	1100
	5022	011000	???	0	??	110000	01	????	0100	0010?00	010	1100
	RJ	011000	???	0	??	110000	01	????	0100	0010?00	010	1100
	PAL	011000	???	0	??	110000	01	????	0100	0010?00	010	1100
	V1	011000	???	0	??	110000	01	????	0100	0010?00	010	1100
T. suis	TSM93	010000	100	0	10	110000	01	0010	0100	0001100	010	1100
	30167	010000	100	0	10	110000	01	0010	0100	0010100	010	1100
	30169	010000	100	0	10	110000	01	0011	0100	0010100	010	1100
Monocercomonas		010010	010	1	01	??????	01	????	0001	0000001	010	1010

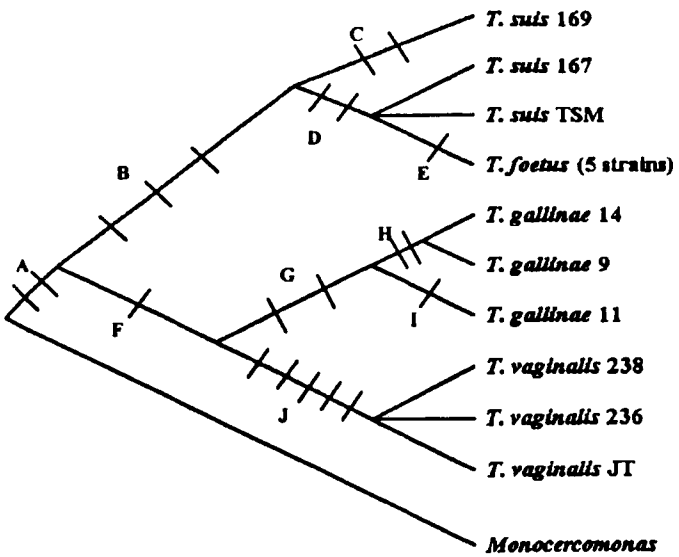


Fig. 5 Strict consensus tree built from the five most parsimonious trees found by PHYLIP. Consistency index (CI) = 0.75

light microscopy (Hibler et al. 1960) and biochemical and antigenic analysis (Robertson 1960; Stepkowski 1966) have suggested that they might be the same species. However, this is the first time that more discriminating methods such as isozymic and electron microscopy have been used.

T. suis shares many fine-structural features with *T. foetus*. Both present the same shape, size, and organelle and cytoskeleton distribution. The size of *T. suis* cultures can vary between 11 and 13.5 μm (Buttrey 1956; Hibler et al. 1960) in work with fixed cultures and measure 17.9 (15–22.5) X 7 (4.5–10) μm in living specimens (Kirby 1951), which are similar to the measurements we obtained for both *T. foetus* and *T. suis*. No morphological or size difference was found among the strains of *T. suis* isolated from either the nasal cavity or the digestive tract of swine. Under electron microscopy, *T. suis* and *T. foetus* could not be differentiated; the mastigont system, the origin and periodicity of the costae, the presence of the comb, and the localization and ultrastructural features of the hydrogenosomes as well as all the other cellular structures were identical to those previously reported for *T. foetus* (Honigberg et al. 1971; Benchimol et al. 1982, 1996; Benchimol and De Souza 1983). The ultrastructural localization of carbohydrates using the Thiéry (1967) technique was also the same in both species as reflected by the arrangement and distribution of glycogen granules and the positive reaction in the same membranous structures.

Among the ultrastructural features shared by the two species are some unreported findings; the thin filamentous bridges connecting the proximal and distal parts of the undulating membrane had previously been suggested by other authors but had never been shown before in thin sections. Also, the bridges connecting the parabasal filament with the first cisternae of the Golgi complex are depicted herein for the first time.

The comb and the origin and periodicity of the costae as well as the number and relationship of the flagella with the cellular body are elements of considerable taxonomic importance when morphological features are considered. In the present study, *T. suis* and *T. foetus* were compared for the first time under electron microscopy. They were also characterized using isozyme electrophoresis and compared with other trichomonads by a cladistic analysis.

Phylogeny of trichomonads based on partial sequences of large-subunit rRNA and on cladistic analysis of morphologiacal data had previously been performed by Viscogliosi et al. (1993), demonstrating that the trichomonads are a monophyletic group that branches very early in the eukaryotic tree. Currently, a number of analytical techniques such as pulsed-field gel electrophoresis are used to determine the degree of genomic diversity among different species. The examination of a

number of strains from related species was performed by Taylor et al. (1994) using genomic DNA from 15 strains of *Heliobacter mustelae*, demonstrating that this species lacks the genomic diversity observed in *H. pylori*.

In comparisons of outcrossing sexual populations of a given organism, the presence of a large number of fixed differences is proof of speciation (Thorpe and Solé-Cava 1994). Because of the inability to apply the biological species concept to asexually reproducing organisms, it is important that a common yardstick be found to estimate how much difference is necessary before two strains are arbitrarily assigned to different species (Avise 1994). One way to do this is to compare the magnitude of the difference between the putative species with that found between conspecific strains in the same group. The magnitude of the isozyme differences found between *T. suis* and *T. foetus* was no larger than that observed between different strains of *T. suis* or *T. gallinae* (but greater than that found in the remarkably homogeneous *T. foetus*). Therefore, we can conclude that the observed differences may well simply represent intraspecific variation. This, coupled with striking ultrastructural and cytochemical similarities, strongly supports the conclusion of other authors (e.g., Hibler et al. 1960; Honigberg 1978; Pakandl and Grubhoffer 1994) that *T. foetus* and *T. suis* belong to the same species. Subtle differences between *T. suis* and *T. foetus* have been found for antigenic reactions (Sanborn 1955) and carbohydrate metabolism (Doran 1957). However, those differences are of the same level as those usually found between conspecific strains rather than between strains from different species.

The presence of the same species of parasite in both cattle and pigs could be the result of a secondary invasion from one host to the other. Given the environmental conditions under which the livestock are kept, a reasonable scenario could be the infection of the nasal cavities of pigs eating in dirty places where contaminated cows had been present.

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