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# Preliminary Characterization of a *Tritrichomonas foetus*-like Protozoan Isolated from Preputial Smegma of Virgin Bulls

**R. H. BonDurant,**[a] *DVM,* **A. Gajadhar,**[b] *PhD,* **C. M. Campero,**[c] *DVM, PhD,* **E. Johnson,**[d] *DVM, PhD,* **Z-R. Lun,**[b] *PhD,* **R. W. Nordhausen,**[e] *MA,* **K. A. Van Hoosear,**[a] *MA,* **M. R. Villanueva,**[e] *BS,* **R. L. Walker,**[e] *DVM, PhD*
[a]*School of Veterinary Medicine, University of California, Davis, CA 95616*
[b] *Centre for Animal Parasitology, Canadian Food Inspection Agency, Saskatoon, Saskatchewan*
[c]*Instituto Nacional de Tecnologia Agropecuaria, INTA-CONICET, Patologia Veterinaria, CC276 (7620) Balcarce, Argentina*
[d]*Veterinary Medical Teaching Hospital, University of California, Davis, CA 95616*
[e]*California Veterinary Diagnostic Laboratory System, University of California, Davis, CA 95616*

## Abstract

Trichomonad isolates from 14 virgin beef bulls in 3 California herds were submitted to the California Veterinary Diagnostic Laboratory for confirmation of a diagnosis of bovine trichomoniasis. Specimens were submitted in self-contained diagnostic pouches and produced cultures with a protozoan suggestive of *Tritrichomonas foetus*. Under bright-field microscopy, large numbers of single-celled motile organisms, approximately 12 µm x 9 µm, with multiple anterior flagellae, a posterior flagellum, axostyle, and a clearly visible undulating membrane were detectable. Motility was jerky and rolling. Air-dried smears of cultures stained with Giemsa or Diff-Quick/iodine revealed an organism similar to *T. foetus,* although somewhat more rounded (less spindle-shaped). Several organisms appeared to have 4 flagellae, and there were numerous dark-staining bodies in the cytoplasm. Scanning electron micrographs (5000 X) clearly showed four anterior flagellae and an undulating membrane on most organisms, and an axostyle that was consistently longer than that seen in *T. foetus*. Specific primers for *T. foetus* were used in a polymerase chain reaction (PCR) assay. No amplification product was detected from any of the "virgin bull" isolates, while positive control isolates of *T. foetus* all yielded an amplicon of the expected size. On the basis of this partial morphological and molecular characterization, we have tentatively concluded that the isolated organism is not *T. foetus*, and speculate that it may be a commensal organism of the lower bowel. The recovery of non *T. foetus* trichomonads in a commercially available diagnostic system suggests that more specific methods may be needed for discrimination among trichomonads isolated from the preputial cavity of bulls.

## Introduction

Bovine trichomoniasis, caused by the protozoan *Tritrichomonas foetus*, is a sexually-transmitted disease characterized by embryonic and fetal loss, and occasional post-coital pyometra.[1] Bulls are chronic carriers of the etiologic agent, while infected cows generally clear the infection from their reproductive tracts within 1-5 months after exposure.[12,13,15] After exposure, bulls older than three years are more likely to become chronically infected than younger bulls,[1,9,13] due to their deeper epithelial crypts of the penis and prepuce, which apparently provide a niche for the parasite.[2] Because transmission is thought to be strictly venereal, all virgin bulls are considered to be uninfected. In the bull, the "gold standard" diagnostic method for many years has involved the scraping or washing of the preputial and penile epithelium, and inoculation of the aspirated material into a selective growth medium.[5,6,14]

Various undefined media, including trypticase-yeast extract-maltose (TYM) medium, modified Diamond's medium, and more recently, the InPouch™[a] system have been shown to nurture the growth of *T. foetus* at 98.6° F (37° C) in aerobic culture. Growth is

[a] Biomed Diagnostics, San Jose, CA

usually seen within 1-7 days.[1,2,6,14,16,17] Cultures are declared positive when a spindle-shaped, motile protozoan flagellate of approximate dimensions 10-25 x 3-15μm, showing characteristic rolling, jerky type of motility, is detected.[10] Morphological characteristics that confirm *T. foetus* include the presence of 3 anterior flagellae (approximately 6-12 μm in length), one posterior flagellum, and a refractile "undulating membrane" that runs almost the entire length of one lateral edge of the protozoan. Although these determinations are typically made under brightfield microscopy at 100-400 X, reliable identification of motile organisms is difficult. Stained preparations could improve morphological assessment, but many of the stains commonly used in practice, such as eosin, nigrosin, or new methylene blue, do not enhance diagnostic value. The sensitivity of the standard (live organism) diagnostic method has been estimated to be approximately 82%, with an assumed specificity of 100%.[14,16] That is, any isolates that appear to meet the morphology and motility criteria are considered to be *T. foetus*.

The objective of this study was to apply recently developed laboratory methods to determine whether trichomonads isolated from the prepuce of virgin bulls during routine screening examinations were in fact *T. foetus*.

## Materials and Methods

Recently, the California Veterinary Diagnostic Laboratory (CVDL) received inoculated InPouches™ from 26 bulls from 3 different beef ranches/facilities in California. The 3 facilities submitted 1, 9, and 16 samples, respectively. The samples were submitted to CVDL to confirm a tentative diagnosis of trichomoniasis. According to the histories, the bulls were virgins, and were less than 24 months of age. In all cases owners stated emphatically that bulls had been raised in isolation from mature females following weaning from their dams at 7 months of age. All bulls were raised in the presence of other bulls. From two facilities, practitioners had inoculated and read culture packets themselves, and forwarded suspect positive cultures to CVDL, while the original samples from the third facility were sent directly to CVDL. All InPouches™ were incubated at 98.6° F (37° C) for up to 7 days, during which time they were examined daily by brightfield microscopy (100X screening; 400X final examination). Cultures were declared tentatively positive on the basis of detecting organisms with the apparent morphology and motility described above. Positive cultures were subcultured in a second InPouch™, TYM, Diamond's medium with sheep serum (DSS), and Diamond's medium with fetal bovine serum (DFBS). Confirming tests included a simple staining of air-dried smears, scanning electron microscopy, and polymerase chain reaction (PCR) assay of specific DNA sequences (described below).

## Confirming Tests, Results and Discussion

Of the 26 pouches submitted from the 3 facilities, 14 (1/1, 1/9, and 12/16, respectively) contained live trichomonads of a size, shape, and motility pattern compatible with *T. foetus*. Initially, survival in InPouches™ was very good. However on subculture, only 7 of these 14 isolates grew well (3+ growth on a subjective scale of 1 to 3) in TYM. The other media supported growth poorly or not at all. Even in TYM, numbers of organisms dwindled with subsequent passages. Only a few isolates survived up to 10 passages, and then only with poor growth. These isolates are currently maintained on Schneider's eggshell medium. In contrast to the slow growth of the suspect organism, "typical" *T. foetus* grows readily *in vitro* and has a doubling time of about 4-5 hours in TYM under optimal conditions.

Approximately 50μl of well-mixed 48-hour inoculum was smeared on each of two glass slides, air dried, and stained with Giemsa, or Diff-Quick® and iodine/iodide, the latter according to Lun and Gajadhar.[11] In virtually all smears, somewhat rounded organisms with multiple anterior flagellae were readily apparent, as was an undulating membrane. A long posterior flagellum and long axostyle were also visible, as were multiple dark-staining, spherical cytoplasmic inclusions (Figure 1). Scanning electron microscopy of 3 of the suspect cultures showed a rounded organism, approximately the same length as *T. foetus*, with a distinctive undulating membrane, a long posterior flagellum and axostyle, and 4 or more anterior flagellae (Figure 2).

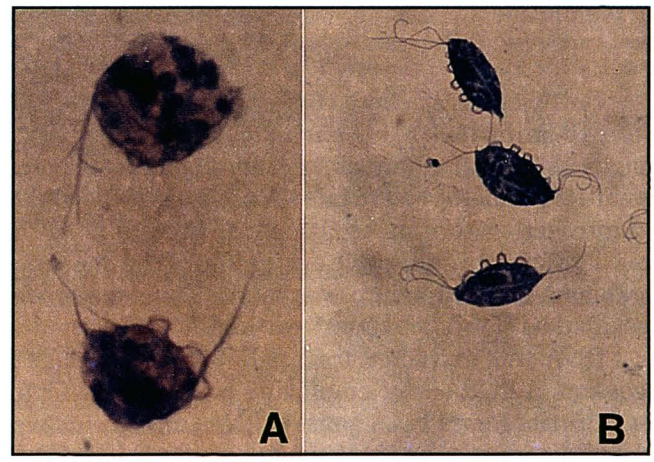


**Figure 1.** **(a)**: Diff-quick/iodine stain of virgin bull isolate (400 X). Note undulating membrane and the multiple, dark-staining spherical inclusions in the cytoplasm of the virgin bull isolate; (**b**): D1 strain of *T. foetus* (400 X). Undulating membrane is clearly seen.

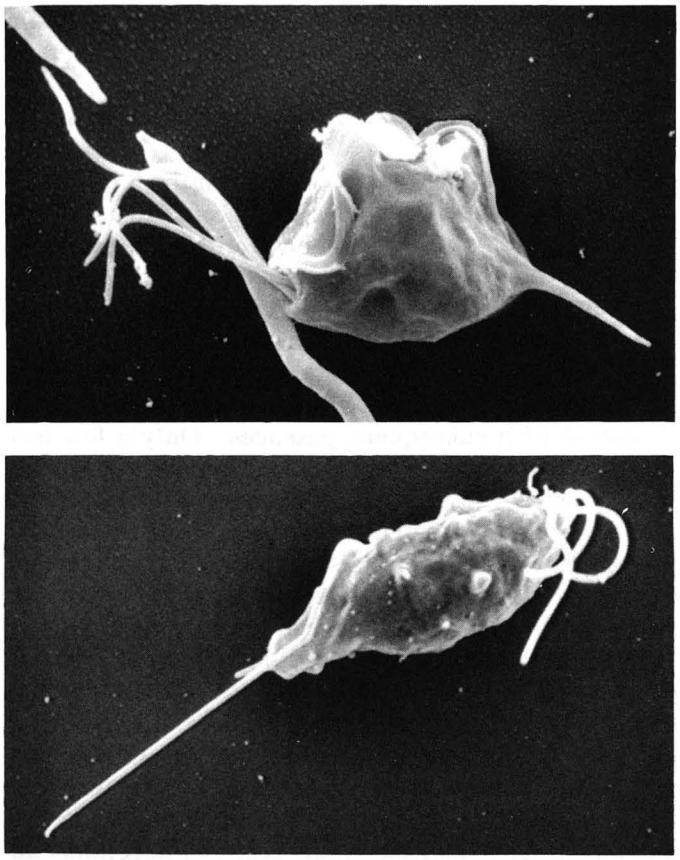

**Figure 2.** Scanning electron micrographs (5000 X) **(top)**: Isolate from prepuce of virgin bull. Note four anterior flagellae, undulating membrane, and long, pointed axostyle. (The posterior flagellum is not visible in this view.) The thick, elongated, tube-like structure behind the anterior flagellae is an artifact; **(bottom)**: D-1 reference strain of *T. foetus*. Note spindle shape, three anterior flagellae, one posterior flagellum, and undulating membrane.

Cultures were pelleted by centrifugation at ~ 2000 g for 10 minutes in a bench-top centrifuge. After resuspension in fresh medium, a small aliquot was frozen in 10% dimethyl sulfoxide (DMSO) at -196°C in liquid nitrogen, according to Campero's protocol.[3] At the time of freezing a subjective evaluation of the density of organisms in the medium was made. The density was classified as "low", "medium", or "high".

DNA was extracted from pellets by proteinase K digestion. A PCR assay, employing primers specific for the 5.8S ribosomal RNA genes (rRNA) of *T. foetus* was used to determine the identity of the protozoan, according to Felleisen.[7,8] The reaction was stopped after 40 cycles and DNA bands were detected by electrophoresis on 2% agarose gels and staining with ethidium bromide. Nucleic acid from isolates of pathogenic *T. foetus* (D-1 strain, as well as isolates from outbreaks of trichomoniasis) were used as positive controls, while DNA from the human parasite, *Trichomonas vaginalis*, was used as a negative control. None of the cultures from suspect cases yielded an amplification product, regardless of the relative cell density in the sample at the time of DNA extraction. In contrast, the D-1 isolate and isolates from outbreaks of trichomoniasis yielded positive PCR products of approximately 347 base pairs in size. Nucleic acid from *T. vaginalis* yielded no amplification product (Figure 3; positive amplicon shown only for D-1 isolate). Representative samples were sent to the Centre for Animal Parasitology in Saskatoon for confirmation of results. At that laboratory, using a Canadian positive reference strain of *T. foetus* and the CVDL virgin bull isolates, identical results were obtained (data not shown).

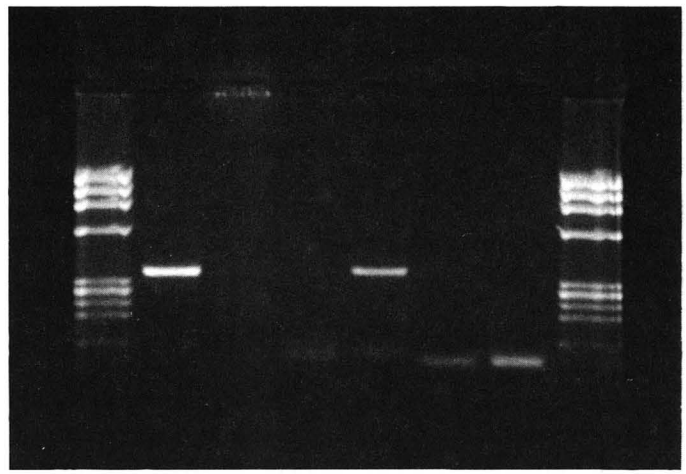

**Figure 3.** Ethidium-stained agarose gels of DNA from different trichomonad isolates after PCR, using primers specific for 5.8s rRNA gene of *T. foetus*. Lane 1 = base-pair markers; Lane 2 = D1 *T. foetus*; Lane 3 = CVDLS virgin bull isolate "a"; Lane 4 = CVDLS virgin bull isolate "b"; Lane 5 = D1 *T. foetus*; Lane 6 = *Trichomonas vaginalis*; Lane 7 = water blank; Lane 8 = base pair markers. The *T. foetus* amplicon is approximately 347 base pairs in size.

## Discussion

Our tentative conclusion is that this organism is not *T. foetus*, but rather a trichomonad protozoan with morphologic characteristics similar to *T. foetus* that can be isolated from the prepuce and penis of young, presumably virgin bulls. The morphological characteristics of this organism bear some similarity to descriptions of gastrointestinal trichomonads, including *Tetratrichmonas pavlovi* and *Tetratrichomonas buttreyi*.[4,9,10] Further characterization is required before precise speciation can be accomplished.

The presence of this trichomonad in the smegma of reported virgin bulls suggests that it may not be heterosexually transmitted. It may be one of the intestinal trichomonads mentioned above. Because young bulls frequently mount and sodomize each other, it is likely that contamination of the penile and preputial epithelium occurs during such behavior. Whether the organism thrives or simply survives in the preputial environment is not known at this time, nor are the detailed growth characteristics of the organism in media that are currently employed for selective growth of *T. foetus*. Subjectively, most of the "virgin bull isolates" appear to grow slowly, if at all, in the standard selective media used for *T. foetus* diagnosis. Only a few isolates appear to be able to grow well in culture.

Although additional diagnostic procedures (special staining, scanning electron microscopy, PCR) ruled out the possibility that this organism is *T. foetus*, it can be easily mistaken for *T. foetus* at the brightfield microscopy level. Thus, the diagnostic procedures used by many practitioners and diagnostic laboratories may not have a specificity of 100%, as previously assumed. Further characterization of the biology and prevalence of this organism or family of organisms is warranted. In the meantime, it would seem prudent to have properly-equipped diagnostic laboratories run more specific, confirmatory tests on suspect cultures, especially those from supposedly virgin bulls. In particular, practitioners encountering suspect cultures may not want to rely solely on light microscopic examination of InPouches™ to make a final diagnosis, but rather they might want to submit suspect cultures for confirmatory tests. The most economical test would be Diff-Quick/iodine staining of smeared cultures, but low numbers of organisms from poorly growing cultures may make it difficult to obtain repeatable results. More specific tests include scanning electron microscopy and the PCR test. The latter is only now becoming available at some diagnostic laboratories.

This non-specific trichomonad was detected because the history did not fit the tentative diagnosis. That is, the isolates were from reported virgin bulls, and therefore by definition should not be the sexually transmitted *T. foetus*. Even if there had been sexual contact with *T. foetus*-infected females, the young age of these bulls makes it unlikely that such a high proportion of them (12 of 16 at one facility) would remain infected long enough to be detected.[1,5,9] It is not known how many diagnoses of trichomoniasis in mature bulls may actually represent false positive results, since heretofore we have assumed that our culture diagnostic test had a specificity of 100%. Research is needed to determine how often, if at all, such false positives occur in mature bulls. In addition, research is needed to determine whether this "virgin bull isolate" can be sexually transmitted, and affect fertility.

## Conclusion

It is possible to obtain a false positive diagnosis of trichomoniasis, at least in virgin bulls, when the diagnosis is based on InPouch™ culture alone. However, the InPouch's high sensitivity and excellent shelf life/convenience make it an ideal "screening" diagnostic test. Confirming tests with higher specificity offer the prospect of distinguishing "true" *T. foetus* from commensal organisms.

## References


1. BonDurant RH: Pathogenesis, diagnosis and management of trichomoniasis in cattle. Vet. Clin. N. Am. (Food Anim. Pract.) 13(2): 345-361, 1997.
2. BonDurant RH, Honigberg BN: Trichomonads of veterinary importance. *In*: Kreier JP, (ed.): Parasitic Protozoa, 2nd ed., San Diego, Academic Press, pp. 111-188, 1994,
3. Campero CM:Use of DMSO for the cryopreservation of *Tritrichomonas foetus* in liquid nitrogen.Vet. Parasitol. 31:339-343, 1989.
4. Castella J, Múnoz E, Ferrer D, Gutiérrez JF: Isolation of the trichomonad *Tetratrichomonas buttreyi* (Hibler et al., 1960) Honigberg, 1963 in bovine diarrhoeic faeces. Vet. Parasitol. 70:41-45, 1997.
5. Clark BL, Parsonson IM, Dufty JH: Experimental infection of bulls with *Tritrichomonas foetus*. Aus. Vet. J. 50: 189-191, 1974.
6. Diamond LS: Lumen dwelling protozoa: *Entamoeba,* trichomonads, and *Giardia*, in Jensen JB (ed.): *In Vitro* Cultivation of Protozoan Parasites. Boca Raton, FL, CRC Press, Inc. pp. 65-109, 1983.
7. Felleisen R: Comparative sequences analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitol. 115: 111-119, 1997.
8. Felleisen R, Lambelet N, Bachmann P, et al.: Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. J. Clin. Microbiol. 36: 513-519, 1998.
9. Honigberg BN: Trichomonads of veterinary importance: *Tritrichomonas foetus*. *In* J. Kreier (ed.): Parasitic Protozoa, 1st ed., New York, Academic Press, pp 207-273, 1978.
10. Levine ND: Flagellates: The Trichomonads, in Veterinary Protozoology, Ames, Iowa, Iowa State University Press, pp. 59-79, 1985.
11. Lun Z-R, Gajadhar AA: A simple and rapid method for staining *Tritrichomonas foetus* and *Trichomonas vaginalis*. J. Vet. Diagnostic Investigation 11: 469-472, 1999.
12. Parsonson IM, Clark BL, Dufty J: Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. J. Comp. Pathol. 86:59-66, 1976.
13. Parsonson IM, Clark BL, Dufty J: The pathogenesis of *Tritrichomonas foetus* infection in the bull. Aus. Vet. J. 50:421-423, 1974.
14. Schönmann MJ, BonDurant RH, Gardner IA, et al.: Comparison of sampling and culture methods for the diagnosis of *Tritrichomonas foetus* infection in bulls. Vet. Rec. 134:620-622, 1994.
15. Skirrow SZ, BonDurant RH: Induced *Tritrichomonas foetus* infection in beef heifers. J. Am. Vet. Med. Assoc. 196:885-889, 1990.
16. Skirrow SZ, BonDurant RH, Farley J, Correa J: Efficacy of ipronidazole against trichomoniasis in beef bulls. J. Am. Vet. Med. Assoc. 187: 404-401, 1985.
17. Thomas MW, Harmon WM, White C: An improved method for the detection of *Tritrichomonas foetus* infection in bulls. Agri-Practice 1:13-17, 1990.

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