Two-step (culture and PCR) diagnostic approach for differentiation of non-\textit{T. foetus} trichomonads from genitalia of virgin beef bulls in Argentina

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Abstract

Preputial fluids from 567 virgin Angus and Hereford bulls, 1–2 years old, were inoculated into Sutherland medium, and approximately 8.4\% produced cultures with a protozoan suggestive of \textit{Tritrichomonas foetus}. Under brightfield microscopy, large numbers of single-celled motile organisms with multiple anterior flagellae, a posterior flagellum, axostyle, and a visible undulating membrane were detectable. Motility was jerky and rolling, as described for \textit{T. foetus}. Air-dried smears of cultures stained with Giemsa or Diff-Quick + iodine revealed an organism similar to \textit{T. foetus}, although somewhat more rounded. Several organisms appeared to have four anterior flagellae. Scanning electron microscopy (5000×) of representative samples revealed four anterior flagellae on most organisms, and an axostyle that was consistently longer than that seen in \textit{T. foetus}. Using pan-trichomonal primers and \textit{T. foetus}-specific primers in a polymerase chain reaction (PCR) assay, amplification products of 372 bp were detected in all virgin bull isolates, but only with the pan-trichomonal primers. Positive control isolates of \textit{T. foetus} yielded amplification products of the expected size (372 and 347 bp) with the two sets of primers, respectively. We conclude that these protozoa are not \textit{T. foetus}, and note the similarity of these findings with those reported earlier in North American beef cattle. Because in several countries there is no legal treatment for bovine trichomonosis, veterinarians recommend slaughter of bulls with positive preputial cultures. The existence of easily mis-identified non-\textit{T. foetus} trichomonads in the bovine prepuce suggests that...
1. Introduction

Argentina is the world’s fourth largest producer of beef and veal, with a national stock of 50.5 million live beef cattle, and an annual production of nearly 3 million metric tons of beef (FAO, 2001). Nearly all beef cattle are raised on pasture, and bred by natural service. In such an industry, pregnancy losses due to reproductive diseases have an important economic impact. Bovine trichomoniasis (trichomonosis), caused by *Tritrichomonas foetus*, is an important disease responsible for low weaning rates in many areas of the country (Cobo and Campero, unpublished diagnostic laboratory information). This sexually-transmitted disease produces reproductive failure due to embryonic and fetal loss. Asymptomatic bulls are the chronic carriers of the etiologic agent (Clark et al., 1974; Parsonson et al., 1974), while heifers and cows generally shed the organism after 5–20 weeks (Skirrow and BonDurant, 1990). Because transmission of the agent is thought to be strictly limited to the venereal mode, virgin bulls are by definition considered to be free of infection. However, in the USA, trichomonads have recently been detected in cultures of preputial scrapings from virgin bulls (BonDurant et al., 1999). In spite of several physical similarities to *T. foetus*, further testing demonstrated that these trichomonads were not *T. foetus*. This paper describes the isolation of non-*T. foetus* trichomonads from preputial scrapings of virgin bulls from a commercial bull stud in Argentina.

2. Materials and methods

2.1. Animals and samples

At a commercial bull stud in the Pampas area of north-central Argentina, virgin bulls of Angus or Polled Hereford breeding were routinely screened for trichomonosis, by culture of preputial scrapings. Young bulls were maintained constantly on pasture, in units of approximately 40 bulls. Preputial scrapings were taken from a group of 84 two-year-old virgin bulls (Group 1) in November 2000. A second group of 483 virgin bulls, mostly yearlings (Group 2), was sampled in March 2001.

Preputial samples were collected by scraping a sterile Cassou artificial insemination sheath back and forth along the surface of the penis and internal prepuce near the fornix. Gentle aspiration was applied during the scraping, and the smegma collected was rinsed into a 3 ml tube containing approximately 1 ml of phosphate-buffered saline (PBS, pH 7.2), by gentle, repeated aspiration and discharge of the PBS (Campero et al., 1992). An aliquot (~0.2 ml) of well-mixed PBS-sample suspension was immediately inoculated into Sutherland’s modified plastridge medium (Sutherland et al., 1953). The inoculated media and the PBS suspensions were sent to a veterinary diagnostic laboratory (Instituto
Azzarini, Lincoln, Buenos Aires Province). Samples arrived at the laboratory on the same day on which they were collected. PBS suspensions were immediately centrifuged (1500×g, 10 min), the supernatants were discarded, and resuspended pellets were examined by direct brightfield microscopy, and then inoculated into Sutherland medium. Both the on-farm-inoculated culture media and the culture media inoculated with resuspended pellets from the transport (PBS) media were incubated at 37 °C in an aerobic incubator. Cultures were maintained for 7 days, and examined microscopically (100× screening; 400× confirmation) each day. Positive samples were declared when the culture showed organisms in the size range of 10–20 to 5–12 μm, with evidence of multiple anterior flagellae, an axostyle, an undulating membrane, and characteristic rolling, “jerky” motility (BonDurant, 1997; BonDurant and Honigberg, 1994).

Presumptive positive culture samples were sent to the government veterinary diagnostic laboratory at INTA, Balcarce, for subculture and further characterization. Other media, including liver infusion broth, trypticase yeast extract maltose (TYM) medium (Diamond, 1983), InPouch® (Biomed Diagnostics, San Jose, CA, USA; see Borchardt et al., 1992) thioglycolate broth, and Schneider’s eggshell medium (Schneider, 1942) were occasionally used in attempts to subculture isolates (BonDurant et al., 1999).

2.2. Staining

A small aliquot (approximately 100 μl) was aspirated from the bottom of tubes containing positive cultures, smeared on a glass slide, and stained with either Giemsa or Diff-Quick + iodine (Merck), as described earlier (Lun and Gajadhar, 1999; BonDurant et al., 1999). Dried, stained smears were examined by brightfield microscopy at 400× and 1000×, and morphology of trichomonad organisms was noted.

2.3. Electron microscopy

Representative positive cultures were processed for scanning electron microscopy, using protocols modified from Benchimol et al. (1990) and employed in an earlier study (BonDurant et al., 1999).

2.4. PCR assay

A polymerase chain reaction (PCR) assay, employing primers specific for sequences in the 5.8S ribosomal RNA gene and flanking internal transcribed spacer regions (ITS) of trichomonads was performed on all “positive” cultures, as described by Felleisen and colleagues (Felleisen, 1997; Felleisen et al., 1998). We used two pairs of commercially synthesized primers (Olitogo, Cruachen, Fisher Scientific, Pittsburg, PA, USA): one pair (TFR 1–2) amplifies a 372 bp segment common to many trichomonad species, while the other pair (TFR 3–4) amplifies a 347 bp product that is specific for T. foetus (Felleisen, 1997). Primer sequences are as follows:

TFR 1: 5′-TGCTTCAGTTCAAGCGGTCTTCC-3′
TFR 2: 5′-CGTAGGTGAAACCTGCCGTGG-3′
TFR 3: 5′-CGGGTTCTTCTATATGAGACAGAACCC-3′
TFR 4: 5′-CCTGCCGTGGATCAGTTCCCTTTGCTTGA-3′
Positive cultures were centrifuged ($1500 \times g$, 10 min), and DNA was extracted from the pellet by proteinase K digestion (Felleisen, 1997). Nucleic acid from pathogenic D1 isolate of T. foetus (originally isolated from an outbreak of trichomonosis, minimally passaged in culture and cryopreserved at $-196^\circ C$; see BonDurant et al., 1993; Campero, 1989) was used as the positive control for all PCR assays, while DNA from the human venereal pathogen, Trichomonas vaginalis, was used as a negative control. The assay was run through 40 cycles of denaturing, annealing, and extension; amplified products and base pair size markers were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed using ultraviolet light transillumination. Because the PCR products of the TFR 1–2 and TFR 3–4 pairs of primers are of similar size (372 and 347 bp, respectively), separate PCR and electrophoretic procedures were conducted for the amplicon of each primer pair.

3. Results

3.1. Prevalence of trichomonads

From 84, Group 1 bulls first sampled in November 2000, seven (8.3%) were diagnosed as “culture-positive” in the first sampling. When resampled 5 weeks later (Table 1), three of these seven (42.9%) showed a second positive culture. Six of the original seven positive bulls were available for a third and fourth sample at 5 and 6.5 months, respectively, following the initial diagnosis, at which times they were all negative. Of 483, Group 2 bulls first sampled in March 2001, 41 (8.5%) were culture-positive for trichomonads. When sampled 10 days

<table>
<thead>
<tr>
<th>Bull no.</th>
<th>Culture resultsb</th>
<th>PCR resultsc</th>
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<tbody>
<tr>
<td>Group 1a</td>
<td></td>
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</tr>
<tr>
<td>X605</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>X631</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td>X667</td>
<td>Positive</td>
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<tr>
<td>623</td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>631</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>687</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>723</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Group 2d</td>
<td>–</td>
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Na: not available for re-sampling.

a 7/84 (8.3%) of sampled bulls were culture positive at first sampling.

b For samples inoculated into culture medium immediately, on-farm. Samples cultured after transport to laboratory in PBS were all negative after incubation in culture media.

c PCR for positive cultures only. See text for description of TFR 1–2, and TFR 3–4.

d 41/483 (8.5%) of sampled bulls were culture positive at first sampling.
later, 6 of these 41 (14.6%) yielded a second positive culture (Table 1). In both years, positive results were detected only from samples that were inoculated into culture media immediately, i.e. on-farm; neither direct microscopy nor culture of the PBS suspensions at the time of arrival at the laboratory yielded a positive result. Subculture of “positive” samples was in general unsuccessful, with the exception of some of those samples subcultured on eggshell medium. Isolates subcultured on thioglycolate or TYM did not survive 48 h, and only a few survived that long, without growth, when inoculated into InPouch® or liver infusion broth. With the latter two media, bacterial overgrowth was common.

3.2. Morphology

The morphological characteristics of the protozoa isolated were similar to those described by BonDurant et al. (1999) for a North American “virgin bull isolate.” Briefly, under bright-field microscopy, motile organisms with multiple anterior flagellae, a posterior flagellum, axostyle, and a clearly visible undulating membrane were detectable. Motility was jerky and rolling. Stained smears of cultures showed an organism similar to T. foetus, although somewhat more rounded, i.e. less spindle-shaped. Several organisms appeared to have four or more anterior flagellae. Examination by SEM (5000×; see Fig. 1) clearly showed four anterior flagellae on most organisms, diverging from a common stalk, which entered the periflagellar canal. In most organisms viewed, the axostyle was longer than that typically seen in T. foetus.

3.3. PCR

All positive cultures from test bulls showed a 372 bp amplicon following PCR with primers TFR 1–2, and no amplicon when primers TFR 3–4 were used. In contrast, positive

Fig. 1. Scanning electron micrograph of a non-T. foetus trichomonad isolated from the prepuce of a virgin bull in Argentina. Note four anterior flagellae diverging from a common stalk and the single posterior flagellum. The undulating membrane is discernable on the right side. The axostyle is not seen well in this view. Bar = 10 μm.
control cultures of *T. foetus* showed a 372 bp amplicon with TFR 1–2, and a 347 bp amplicon with TFR 3–4. Negative control *Trichomonas vaginalis* cultures yielded only the TFR 1–2 generated band, slightly larger than 372 bp (see Fig. 2).

4. Discussion

The significance of the finding of the non-*T. foetus* trichomonads in preputial fluids is related to the fact that in many countries, no legal efficacious treatment exists for trichomonosis in either the bull or cow (BonDurant, 1997). Because of this, and because bulls tend to be chronic carriers of *T. foetus*, the only ethical advice a veterinarian can give to owners of infected bulls is to sell them for slaughter. It is therefore critical that a positive diagnosis be based on a highly specific diagnostic test, i.e. one with a minimum of false positive results. The finding of convincingly motile trichomonads with morphology similar to *T. foetus*—at least at the light microscope level available to veterinary practitioners—indicates that the current “gold standard” diagnostic test, i.e. culture of preputial smegma with microscopic determination of morphology and motility consistent with trichomonads, is not 100% specific, as previously assumed (BonDurant et al., 1990). We suggest that a two-step diagnostic strategy be developed for trichomonosis in bulls. The first step would involve culture of preputial scrapings, a relatively inexpensive procedure that has been shown to be approximately 90% sensitive (Borchardt et al., 1992; Kimsey, 1986; Skirrow et al., 1985). The second step would be PCR confirmation of culture-positive samples. Before this two-step method can be widely applied, a quantitative estimate of the actual specificity of the PCR assay needs to be determined. This will
require the acquisition and testing of a large number of isolates over a wide geographical range.

This study also confirms the findings of non-\emph{T. foetus} trichomonads in bovine preputial scrapings that were first suggested a half-century ago by Morgan and Hawkins (1948), and more recently described in North America by BonDurant et al. (1999), and represents, to our knowledge, the first report of such trichomonads in Argentina. We presume that at least some of these trichomonads are not pathogens, based on our recent observation in California that deep vaginal instillation of $10^6$ live trichomonads of similar morphology (originally isolated from preputial cavities of virgin bulls) into five estrous virgin heifers did not establish infection in any heifers (Borrowman and BonDurant, unpublished observations). This same dose of D1 strain of \emph{T. foetus} reliably infects 100% of estrous virgin heifers (BonDurant et al., 1993).

In our previous report of non-\emph{T. foetus} trichomonads, isolated from the genitalia of North American virgin bulls, we also noted four anterior flagellae, but they entered the periflagellar canal independently, as the three anterior flagellae of \emph{T. foetus} do (BonDurant and Honigberg, 1994; Levine, 1985), rather than on a common stalk. The organism described here is probably of a different species than the one described in North America. This further emphasizes the point that there may be several taxa of trichomonads that can be isolated from the prepuce of bulls.

The origin of the non-\emph{T. foetus} trichomonads was not sought in this study, but it seems highly likely that these organisms are lower bowel residents. Trichomonads of somewhat similar morphology to those reported here have been described in feces of cattle (Castella et al., 1997). Given that young bulls housed together often mount and sodomize each other (Jezierski et al., 1989), it is reasonable to expect that the preputial cavity would become contaminated with fecal trichomonads. Our laboratory has occasionally diagnosed the presence of such non-\emph{T. foetus} trichomonads in mature bulls as well (BonDurant, unpublished observations), so that the two-step method suggested above would be applicable to bulls of all ages.

That two large groups of bulls would show a similar prevalence of 8.3–8.5% may be coincidental, or may be related to mounting frequency, diet, or perhaps the prevalence of genetic susceptibility of host bulls to genital colonization by these trichomonads. Assuming that the repeated cultures yielded the same species of organisms on both occasions, the fact that a second “false positive” culture could be obtained 10 days (March group) or 5 weeks (November group) after the first culture suggests that either these trichomonads can survive for long periods in the preputial cavity, or that re-contamination occurs.

In this study, non-\emph{T. foetus} trichomonads could only be detected when preputial scrapings were directly inoculated into culture medium, i.e. they did not survive the journey to the diagnostic laboratory when suspended in PBS. In a previous study with \emph{T. foetus}, a 14% loss in viability was shown when samples were cultured after overnight suspension in lactated Ringer’s solution rather than being directly inoculated into culture medium (Skirrow et al., 1985). Moreover, our results are compatible with anecdotal reports that non-\emph{T. foetus} trichomonads are difficult to subculture in most trichomonad-supporting media (BonDurant and Honigberg, 1994).

In our previous report on detection of non-\emph{T. foetus} trichomonads from virgin bulls, we used only the specific primers TFR 3–4 (BonDurant et al., 1999). The use of two sets of PCR
primer pairs, one that reacts with a broad spectrum of trichomonads and one that is apparently \textit{T. foetus}-specific, provides a procedural control. That is, if a cultured trichomonad does not yield the 347 bp amplicon following PCR with the (specific) TFR 3–4 primers, the possibility of insufficient DNA/protozoa numbers accounting for the negative results can be ruled out if the non-\textit{T. foetus} TFR 1–2 produces a detectable band of 372 bp after PCR, digestion, and electrophoresis.

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