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Veterinary Parasitology 120 (2004) 145–150
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

Failure to establish infection with *Tetratrichomonas* sp. in the reproductive tracts of heifers and bulls

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Received 1 July 2003; received in revised form 16 December 2003; accepted 23 December 2003

Abstract

Experimental infection of the reproductive tracts of heifers and bulls with *Tetratrichomonas* sp. isolated from preputial smegma of virgin bulls was attempted. Nine heifers and four bulls were challenged by inoculation of 7×10^6 *Tetratrichomonas* sp. into the vaginal lumen and preputial cavity, respectively. Vaginal mucus and preputial smegma samples were collected and cultured for *Tetratrichomonas* sp. Heifers were slaughtered in groups of three at 2, 9 and 21 days after inoculation. Two heifers and two bulls infected with *Tritrichomonas foetus* and two uninfected heifers were used as controls for the model infection. *Tetratrichomonas* sp. were only isolated in vaginal mucus of 7/9 inoculated heifers at 6 h post-inoculation, and genital secretions taken at slaughter time from vagina, uterus and oviduct were cultural negative. Bulls challenged with *Tetratrichomonas* sp. remained cultural negative. Since *Tetratrichomonas* sp. survived only a few hours in the female genitalia and did not survive in the male genitalia after experimental challenge, *Tetratrichomonas* sp. did not colonize the genital tract. These were likely trichomonads from the digestive tract. Collection of clean samples without fecal contamination from the reproductive tract is proposed as a measure to avoid *Tetratrichomonas* sp. transitory genital infection.

Keywords: *Tetratrichomonas* sp.; *Tritrichomonas foetus*; Cattle; Protozoa

1. Introduction

Recently, *Tetratrichomonas* sp. were isolated from preputial smegma of beef virgin bulls (BonDurant et al., 1999; Campero et al., 2003). This protozoan had four anterior flagella of

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doi:10.1016/j.vetpar.2003.12.013

unequal length and a recurrent one forming the undulating membrane, one anterior nucleus, a Golgi complex, an axostyle, and a costa (Cobo et al., 2003). Also, the hydrogenosomes were rather elongated, seen in groups, and vacuoles of different sizes containing bacteria and material undergoing digestion were frequently found (Cobo et al., 2003). Finding motile trichomonads with morphology similar to *Tritrichomonas foetus* in the culture medium represented a serious problem for the diagnosis of bovine tritrichomonosis (BonDurant et al., 1999; Campero et al., 2003). Under diagnostic lab conditions, observing the culture only by brightfield light microscopy, *Tetratrichomonas* sp. and *T. foetus* are so similar that they can only be distinguished by polymerase chain reactions or ultrastructural studies as transmission electron microscopy or scanning electron microscopy (BonDurant et al., 1999; Cobo et al., 2003). It is well known that *T. foetus* establishes a chronic infection, but produces no detrimental effects on libido and sperm quality of bulls, and causes early abortion, infertility due to vaginitis, endometritis and salpingitis in cows (Skirrow and BonDurant, 1988). However, no information about the pathogenesis of *Tetratrichomonas* sp. infection of the reproductive tract is available. The purpose of the present study was to investigate the pathogenesis of *Tetratrichomonas* sp. under experimental conditions in the genitalia of heifers and bulls.

2. Materials and methods

Tetratrichomonas sp. were isolated from the preputial smegma of virgin bulls from a beef herd (Campero et al., 2003). The *T. foetus* strain was originally obtained from a cow with pyometra and a clone from this isolate was used (Cobo et al., 2002). Protozoa were grown at 37 °C in microaerobic atmosphere in liver infusion medium (LI) as was described (Sutherland et al., 1953) with minor modifications (Campero et al., 1986).

Thirteen virgin postpubertal, 18 months old, Aberdeen Angus and Hereford crossbred heifers with normal reproductive tracts obtained from a herd free of Brucellosis and venereal diseases (Trichomonosis and genital Campylobacteriosis) were divided into five groups. Before the study began, to verify that there had been no exposure to *T. foetus*, vaginal cultures for trichomonads were done two times a week apart. To standardize effects of the estrous cycle, females were synchronized to be in estrus at the inoculation time (Estrumate, Cloprostenol, Schering-Plough, Coopers, Germany). Heifers from groups A ($n = 3$), B ($n = 3$) and C ($n = 3$) were challenged by inoculation of 7×10^6 *Tetratrichomonas* sp. in 3 ml of phosphate-buffered solution (PBS) in the cranial part of the vagina as was mentioned (Campero et al., 1993; Corbeil et al., 1998). Heifers of group D ($n = 2$) were similarity inoculated as a positive control by 7×10^6 *T. foetus* in 3 ml of PBS, and heifers from group E ($n = 2$) remained as uninfected controls.

Six 6-year-old Aberdeen Angus bulls assigned a satisfactory breeding status on the basis of physical soundness, scrotal circumference, negative trichomonad culture and semen examination were divided into two groups. Males from group F ($n = 4$) were experimentally infected by intrapreputial inoculation of 7×10^6 *Tetratrichomonas* sp., and those from group G ($n = 2$) by 7×10^6 *T. foetus* as was described above.

Genital fluid samples taken by aspiration from the cranial vagina and from the preputial cavity were collected at 6 h post-inoculation, and then daily until the slaughter time in

heifers and until day 21 after inoculation in bulls. Aliquots were cultured onto LI media with antibiotics (1000 U/ml of penicillin G, 1 mg/ml of streptomycin sulfate and 500 U/ml of nystatin). In animals from groups A–C and F (challenged with *Tetratrichomonas* sp.), genital samples were also collected in PBS for direct microscopic examination as was mentioned (Campero et al., 1993), and cultured in Schneider's eggshell medium (SE). Briefly, this media combine whole eggs, defibrinated cow blood and sodium carbonate solution in slant tube covered by a liquid solution of sodium citrate solution, bovine serum and hematein (pH 7.2–7.3) (Schneider, 1942; BonDurant et al., 1999; Campero et al., 2003).

Heifers from group A were slaughtered at 2 days post-challenge, those from group B at 9 days post-challenge, and those from groups C–E at 21 days post-challenge. At necropsy, genital fluid secretions for trichomonad were collected in the same mediums (LI, SE and PBS), and tissue samples for histological examination were obtained from vagina, glandular inter-caruncular endometrium and ampullar oviduct. The tissues were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 5 μm, and routinely stained with haematoxylin and eosin.

3. Results

To evaluate the viability of the infective inocula, an aliquot of the remnants of the *T. foetus* and *Tetratrichomonas* sp. inocula in PBS were maintained at room temperature and examined daily under light microscopy. Both trichomonad inocula maintained adequate motility until at least 72 h post-inoculation.

Culture of vaginal secretions from *Tetratrichomonas* sp. challenged heifers showed that 7/9 (1/3 of group A, 3/3 of groups B and C, respectively) were positive on culture at 6 h post-inoculation. All positive cultures were positive in both LI and SE media. In addition, only one heifer from group C was also positive for *Tetratrichomonas* sp. on PBS by direct micoscopic observation. Positive cultures in LI were detected between 48 and 120 (mean 72) h of incubation, and SE between 24 and 72 (mean 48) h of incubation. In the samplings after 6 h post-inoculation, no heifer in groups A–C had a positive culture. In the case of bulls from group F (*Tetratrichomonas* sp. challenged) were always cultural negative in LI, SE and PBS mediums. All *T. foetus* inoculated heifers (group D) and bulls (group G) remained infected from 6 h until 21 days post-inoculation, the end of the study. Non-infected heifers (group E) were negative on culture throughout the study.

At slaughter, *Tetratrichomonas* sp. were not isolated from any heifers from groups A, B, or C. In heifers from group D, slaughtered at 21 days post-inoculation, *T. foetus* was isolated from vaginal, cervical, uterine and oviductal secretions. Heifers from group E (non-infected) were slaughtered also at 21 days post-infection, and resulted cultural negative from vaginal, uterine and oviductal samples.

Macroscopic and histological examination of tissue samples of the vagina, uterus, and oviduct of *Tetratrichomonas* sp. inoculated heifers (groups A–C) revealed no histopathological lesions. The *T. foetus* infected heifers (group D) presented non-suppurative endometritis with some focal lymphoid aggregates in the stratum spongiosum, mild vaginitis and mild salpingitis. No gross or microscopic lesions were registered in control heifers (group E).

4. Discussion

After experimental challenge with appropriate system and viable *Tetratrichomonas* sp. did not result in genital infection of heifers and bulls (Cobo et al., 2001; Cobo et al., 2002). Histopathological findings in the genitalia of *T. foetus* infected heifers were consistent with reports of early *T. foetus* infection (Skirrow and BonDurant, 1988) as well as the *T. foetus* isolations throughout the genital fluids of female and male support the effectiveness of the inoculation method.

Non-*T. foetus* trichomonads in preputial fluids, specifically *Tetratrichomonas* sp., have been reported recently in North America (BonDurant et al., 1999) and Argentina (Campero et al., 2003). The significance of this finding needs to clarify because the pathogenicity of the described organisms is not known. To avoid diagnostic confusion, it has been suggested that a two-step diagnostic strategy is employed, including as first step a culture of preputial scrapings and microscopic evaluation (specific flagella stains), and, as the second step, PCR confirmation of culture-positive samples (Campero et al., 2003). Regarding pathogenic significance, although it was believed that these trichomonads are not pathogens (BonDurant et al., 1999; Campero et al., 2003), little information about their taxonomic characterization and pathogenesis in heifers and bulls has been described.

Under the protocol used in the present study, we provide support for the hypothesis that this *Tetratrichomonas* sp. is non-pathogenic, as it did not survive in the preputial cavity and survived only 6 h in the vagina. In order to explain the lack of reproductive tract colonization by this flagellate, some hypotheses could be developed. First of all, the different morphology of this *Tetratrichomonas* sp., showing distinctive ultrastructural features when compared to *T. foetus* (Cobo et al., 2003), could explain the non-invasion. It is known that structural morphology and change from ellipsoid to amoeboid shape determine the attachment of trichomonads such as *T. foetus* and *Trichomonas vaginalis* (Alderete et al., 1995; Petrin et al., 1998; Felleisen, 1999). However, while *T. foetus* adheres firstly by posterior flagella and then by the soma (Alderete et al., 1995; Felleisen, 1999), *T. vaginalis* adheres by the opposite side of undulating membrane where there are more lectin adhesins (Alderete et al., 1995; Petrin et al., 1998). In the present case, the isolated *Tetratrichomonas* sp. have four anterior flagella of unequal length and a recurrent one forming the prominent undulating membrane with three to five waves plus a fast, erratic and non-directional movement (Cobo et al., 2003).

Secondly, the fact that *Tetratrichomonas* sp. only survived few hours in the vagina, could be due to different facts like the presence of not adhesins, enzymes, like cistein proteinases and neuraminidases, which are also involved in the *T. foetus* adherence. However, analyses of *Tetratrichomonas* sp. adhesins or enzymes were not performed in the present study and further research need to be done.

Moreover, highly specific metabolic requirements of trichomonads appear to be necessary for survival of the parasite in the different environment of the bovine reproductive tract, constantly changing with regard to pH, hormones, menses and the nutrient supply (Alderete et al., 1995; Petrin et al., 1998). Vaginal pH influences the adherence of *T. foetus* to bovine vaginal epithelium, which must be between 6 and 7.5 (Thomford et al., 1996), and of *T. vaginalis* to human vaginal epithelium, which must be between 3.8 and 4.2 (Petrin et al., 1998). It is possible that bovine vaginal pH might not be appropriate for *Tetratrichomonas*

sp. Data is lacking with reference to the metabolic requirements and pH preference of this *Tetratrichomonas* sp. to clarify this point. However, the presence of many bacteria adhered to the surface of this flagellate and vacuoles containing bacteria and material in the process of digestion in its cytoplasm (Cobo et al., 2003) suggest a nitrogen metabolism from bacterial digestion. *Tetratrichomonas* sp. isolated from genital of bulls may be lower bowl residents associated with bacteria from digestive tract (BonDurant et al., 1999; Campero et al., 2003). Interaction between trichomonads and bacteria was previously described in salpingitis in Pekin ducks associated with infection of *Tetratrichomonas* sp. and *Escherichia coli* (Crespo et al., 2001), and in women trichomonosis where *T. vaginalis* phagocytoses *Lactobacillus acidofilus*, part of the normal flora (Petrin et al., 1998). The clean environment of the bovine vagina without presence of bacteria as well as the differentiation in the *Tetratrichomonas* sp. metabolic could explain lack of nitrogen source for *Tetratrichomonas* sp., affecting viability and the capacity to colonize in the vaginal environment, as was seen in the present work.

It is supposed that homosexual behavior among bulls and posterior fecal presence in preputial cavity and penis is the route of contamination of this *Tetratrichomonas* sp. (BonDurant et al., 1999; Campero et al., 2003). Moreover, mounting frequency related with young bulls housed together, diet associated with diarrhea, and genetic susceptibility of host bulls to genital colonization by *Tetratrichomonas* sp., it was suggested as a course of this finding (Campero et al., 2003). This fact suggest the importance to obtain a clean preputial sampling avoiding soil, stagnant water, standing urine and feces of animal contamination. This *Tetratrichomonas* sp. probably depends on digestive bacteria and the transitory infection may be related to fecal contamination.

Regarding the taxonomic classification, we hypothesize that this flagellate should be assigned *Tetratrichomonads buttreyi* (Castella et al., 1997; Cobo et al., 2003) considering that the protist had four flagella of unequal length and a prominent undulating membrane. Ultrastructural characterization was not performed for other *Tetratrichomonas* sp. isolations from bulls (BonDurant et al., 1999; Campero et al., 2003). However, similarities in the morphology and culture features, suggest that could be the same specie. Further investigation including ultrastructural studies is needed to investigate weather other trichomonads could colonize the genitalia and their relation with bacteria and genital environment.

In conclusion, this study provides evidence that *Tetratrichomonas* sp. is non-pathogenic for the reproductive tract of bulls and heifers. Considering that this flagellate has origin in the digestive tract and it is associated with bacterial contamination, adequate collection of clean genital samples, avoid erroneous bovine trichomonosis diagnostic.

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