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Tritrichomonas foetus: Prevalence study in naturally mating bulls in Switzerland

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Keywords:
Tritrichomonas foetus
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Tetratrichomonas sp.
Fertility problems
Culture and PCR

Abstract
Switzerland is officially free from bovine Tritrichomonas foetus. While bulls used for artificial insemination (AI) are routinely examined for this pathogen, bulls engaged in natural mating, as well as aborted fetuses, are only very sporadically investigated, indicating that the disease awareness for bovine tritrichomoniasis is low. Natural mating in cattle is becoming increasingly popular in Switzerland. Accordingly, a re-introduction/re-occurrence of T. foetus in cattle seems possible either via resurgence from a yet unknown bovine reservoir, or via importation of infected cattle. The low disease awareness for bovine tritrichomoniasis might favor an unnoticed re-establishment of T. foetus in the Swiss cattle population. The aim of our study was thus to search for the parasite, and if found, to assess the prevalence of bovine T. foetus in Switzerland. We included (1) bulls over two years of age used in natural mating and sent to slaughter, (2) bulls used for natural service in herds with or without fertility problems and (3) aborted fetuses. Furthermore, the routinely examined bulls used for AI (4) were included in this study. In total, 1362 preputial samples from bulls and 60 abomasal fluid samples of aborted fetuses were analyzed for the presence of T. foetus by both in vitro cultivation and molecular analyses. The parasite could not be detected in any of the samples, indicating that the maximal prevalence possibly missed was about 0.3% (95% confidence). Interestingly, in preputial samples of three bulls of category 1, apathogenic Tetratrichomonas sp. was identified, documenting a proof-of-principle for the methodology used in this study.

1. Introduction

The flagellated bovine protist Tritrichomonas foetus (Riedmüller, 1928) is well known as the causative agent of a venereally transmitted disease in cattle that can lead to infertility and abortion (BonDurant, 1997). Apathogenic flagellates like Tetratrichomonas sp. may sometimes be detected in preputial samples of bulls and need to be differentiated from T. foetus by molecular methods (Campero et al., 2003). In Switzerland and other regions where artificial insemination (AI) flanked by rigorous quarantine testing of the bulls used therein is common, the prevalence of bovine tritrichomoniasis and in parallel the awareness for this disease, is generally low. Indeed, the last reported
increased in an extensively managed beef breed has been reported from Spain (Mendoza-Ibarra et al., 2012). To address these concerns, a study was designed to investigate for the first time in Switzerland the presence of the parasite in a sub-population of Swiss cattle most likely to be infected: (1) elderly bulls used in natural service, (2) naturally mating bulls from herds with or without fertility problems, and (3) aborted fetuses. Furthermore, the data of the routine examination of bulls used for AI were included in the study.

2. Materials and methods

2.1. Study design and sample size

The sampling period was January to December 2012. We considered male gender, increasing age of bulls, natural mating, fertility problems in the herd, and abortions as risk factors for, or indicators of, infection with T. foetus (BonDurant, 1997; Rae et al., 2004; Campero and Gottstein, 2007; Sager et al., 2007; Mendoza-Ibarra et al., 2012). Bulls destined for meat production are usually slaughtered within the first two years of life. As we targeted bulls used for reproduction, we aimed at sampling bulls aged over 24 months. The bull-level test sensitivity was assumed as 0.78 and the specificity as 1.0 (Cobo et al., 2007). Thus, the sample size necessary to obtain a system sensitivity of 99% (with 95% confidence) to detect a prevalence of between 0.001 and 0.005 in a population was considered unfeasible, we collaborated with slaughterhouses to target bulls sent for slaughter and available for sampling. In total, 1098 bulls could be sampled by this approach. Additionally, we included samples from 61 live bulls, 14 of these originated from herds with fertility problems such as repeat breeders, embryonic loss, abortions, or increased incidence of chronic clinical endometritis, and also obtained samples from 203 bulls used in AI. This resulted in an effective sample size of 1362 bulls. Additionally, we obtained samples from 60 aborted fetuses.

2.2. Sampling procedure

From bulls sampled at the slaughterhouse, the distal part of the penis including the prepulse and the fornix was collected, sealed in individual plastic bags to avoid loss of moisture, and sent to the Institute of Parasitology in Bern (IPB) using individual plastic containers without cooling. The samples arrived within 24 h at the laboratory. Upon arrival of the samples, the fornix region of the penis/prepulse was scraped with sterile metal scrapers.

Part of the collected material was transferred directly to the InPouch™ system (Biomed diagnostics, San José, CA., USA) and another part was stored at −20 °C for subsequent molecular analyses.

Live bulls used in natural service were sampled by preputial washings using sterile isotonic saline (Schönmann et al., 1994). Those samples were sent in 15 ml Falcon® tubes at ambient temperature and arrived within four to 24 h at the IPB. The sediment was used to inoculate the InPouch™ cultivation system, and for molecular analysis.

Aborted bovine fetuses or abomasal fluid of the fetuses were submitted within 24 h to the IPB without cooling. Upon arrival, fetuses were opened and abomasal fluid was extracted. The abomasal fluid was used to directly inoculate the InPouch™ system, and for molecular analysis.

2.3. In vitro cultivation

All samples were investigated by using the bovine InPouch™ system. The pouches were cultivated in an upright position at 37 °C for three consecutive days (Sager et al., 2007). A positive control pouch was included in each cultivation series. The cultures were controlled for growth of trichomonads by light microscopy (100× magnification) every 24 h. Culture was marked as positive or negative based on the presence of motile flagellated trophozoites. If trophozoites were visible, 100 μl of the culture sediment was used for molecular analysis.

2.4. Purification of genomic DNA and PCRs

Genomic DNA of all samples was extracted following the protocol for cultured cells of the DNeasy® Blood and Tissue Kit (Qiagen, Switzerland). DNA was eluted in 200 μl elution buffer. A panspecific PCR for amplification of the internal transcribed spacer region 1 (ITS1) of various trichomonads was performed as earlier described (Frey et al., 2009). Forward 18S primer (5′-GTAGGTGAACCTGCCGTTG-3′) and reverse 5.8S primer (5′-TTCAGTTCAGCGGGTCTTC-3′) (MWG-Biotech Inc., Germany) were used. For T. foetus, amplification products of 367 bp were anticipated, and for Tetratrichomonas sp. amplification products of 370 to 400 bp were anticipated. To prevent carry-over contamination from previous diagnostic reactions, uracil DNA glycosylase (UDG) and dUTP (instead of dTTP) were included in the reaction mixture (Longo et al., 1990). Briefly, one PCR (total volume 25 μl) consisted of 2 μl DNA, 2.5 μl 10× PCR buffer (Perkin-Elmer, Rotkreuz, Switzerland), 0.2 mM each dATP, dGTP, and dCTP, 0.4 mM dUTP (Amer sham Biosciences), 6.25 pmol each of the primers, 2 units

case of a bull infected with T. foetus in Switzerland dates from 1997 (Anonymous, 1997). Where natural mating is widely practiced, e.g. in Argentina or the U.S.A., bovine tritrichomoniasis is still prevalent (Mardones et al., 2008; Rae et al., 2004; Rodning et al., 2008). In the last decade, beef and dairy herds relying on natural mating have become more and more important in Switzerland. Natural mating is the predominant reproduction type in Swiss beef herds. Accordingly, the number of bulls aged over two years, i.e. bulls used for breeding, has increased by about 30% between 2009 and 2012 (Federal Office for Statistics, 2013). In bulls used for natural mating as well as in aborted fetuses testing for T. foetus is not mandatory (Anonymous, 1995) and therefore only very sporadically performed. The increasing importance of natural breeding in combination with the current low awareness of bovine tritrichomoniasis might lead to an unnoticed re-establishment of the parasite in Swiss cattle. Very recently, such a re-establishment in an extensively managed beef breed has been reported from Spain (Mendoza-Ibarra et al., 2012). To address these concerns, a study was designed to investigate for the first time in Switzerland the presence of the parasite in a sub-population of Swiss cattle most likely to be infected: (1) elderly bulls used in natural service, (2) naturally mating bulls from herds with or without fertility problems, and (3) aborted fetuses. Furthermore, the data of the routine examination of bulls used for AI were included in the study.
of AmpliTaq™ DNA polymerase (Perkin-Elmer) and 0.5 units of uracil DNA glycosylase (UDG; Roche, Switzerland). MgCl₂ was supplemented to a final concentration of 2.5 mM.

Amplification products were visualized by agarose gel electrophoresis (2%) using ethidiumbromide staining. A PCR specific for Tririchomonas sp. (Felleisen et al., 1998) using forward primer TFR4 (5′-CTGCCGTGACATCCGTAA-3′) and reverse primer TFR3 (5′-CGGGCTTTCCTATTAGCACAGAACC-3′) was additionally performed with samples showing a positive in vitro culture and positive panspecific amplification product. One reaction of 25 μl consisted of 2 μl DNA, 2.5 μl 10× PCR buffer (Perkin-Elmer), 0.2 mM each dATP, dGTP, and dCTP, 0.4 mM dUTP (Amersham Biosciences), 6.25 pmol each of the primers, 2 units of AmpliTaq™ DNA polymerase (Perkin-Elmer) and 0.5 units of UDG (Roche). MgCl₂ was supplemented to a final concentration of 2.5 mM.

2.5. Cloning and sequencing

Of all samples of which flagellates were grown in vitro, the ITS1 was amplified from genomic DNA with the high fidelity, proof-reading DNA polymerase phusion (New England BioLabs M0530L) using the primers of the panspecific PCR (forward 5′-GAATTCGACCTGCGCCGGT-3′ and reverse 5′-TGCAGTCAGGCCGCTTCCTC-3′). The PCR product was ligated into pCR-Blunt II TOPO vector (Invitrogen 45-0245) according to manufacturer’s protocol, transformed into chemo-competent Escherichia coli (Top10, Invitrogen) and grown under Kanamycin selection. Plasmid DNA of positive clones was extracted using a QIAprep spin miniprep kit (QIAGEN cat no. 27106) according to manufacturer’s protocol. For dye incorporation, a sequencing reaction using BigDye Terminator (Applied Biosystems 4336699) was used according to manufacturer’s protocol. DNA was ethanol precipitated and taken up in Hi-Di Formamide (Applied Biosciences) for sequencing. A sequencing reaction using BigDye Terminator (Applied Biosystems 4336699) was performed on an ABI3730 sequence analyzer. Sequences were analyzed using the following online tools: Basic Local Alignment Search Tool (nBLAST from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and reverse-complement.com.

2.6. Characterization of sampled bulls

All sampled bulls were identified in the animal data base (Identitas AG, Bern, Switzerland). Origin, age, and breed were recorded and compared with the according data of all bulls older than two years living in 2012 in Switzerland.

2.7. Statistical analyses

For statistical analyses, only the data from bulls aged over two years were included. The target population was all bulls aged over 24 months that lived or were slaughtered in 2012 in Switzerland. The probability of freedom achieved for T. foetus in Swiss bulls was calculated using the “Population Level Sensitivity module” (epitools.ausvet.com.au) with the following inputs: Sample size = 1194, population size = 12,445, sensitivity of test method (culture, PCR, and sequencing) = 1.0, a desired confidence of 0.95 and design prevalences of 0.002, 0.003 and 0.004.

3. Results

3.1. Description of sampled bulls

We obtained the information about the country of origin for all bulls included in our study (n = 1362; 100%). The birth date and breed was recorded for 1357 bulls (99.6%). 163 bulls (11.9%) were younger than two years at the time of sampling. Of these, 134 were bulls used in AI and 23 were naturally mating bulls sampled in their herds. Six bulls sampled at the slaughterhouse were younger than two years. Origin, age, and breed of the bulls aged over three years were slightly over-represented (Table 1). The composition of our sampled bulls mirrored very nicely the target population in terms of origin of the animals, as well as for the age distribution, although in our sample the bulls aged over three years were slightly over-represented (Table 1). In terms of production types, a slight overrepresentation of breeds used in milk production (Red Holstein, Holstein Friesian), or two purpose breeds (Simmental) in the sampled population was observed.

Table 1

<table>
<thead>
<tr>
<th>Origin</th>
<th>Analyzed bulls* (n = 1194)</th>
<th>Target population** (n = 12,445)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Origin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Switzerland</td>
<td>1163 (97.5)</td>
<td>12,159 (97.7)</td>
</tr>
<tr>
<td>France</td>
<td>18 (1.5)</td>
<td>100 (0.8)</td>
</tr>
<tr>
<td>Other Europe</td>
<td>13 (1.1)</td>
<td>187 (1.5)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–3 years</td>
<td>334 (28)</td>
<td>6011 (48.3)</td>
</tr>
<tr>
<td>3–4 years</td>
<td>423 (35.4)</td>
<td>3708 (29.8)</td>
</tr>
<tr>
<td>4–5 years</td>
<td>191 (16)</td>
<td>1195 (9.6)</td>
</tr>
<tr>
<td>5–6 years</td>
<td>111 (9.3)</td>
<td>585 (4.7)</td>
</tr>
<tr>
<td>6–7 years</td>
<td>60 (5)</td>
<td>373 (3)</td>
</tr>
<tr>
<td>7–8 years</td>
<td>39 (3.3)</td>
<td>249 (2)</td>
</tr>
<tr>
<td>8 years or older</td>
<td>36 (3)</td>
<td>336 (2.7)</td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red Holstein</td>
<td>254 (21.3)</td>
<td>1568 (12.6)</td>
</tr>
<tr>
<td>Limousin</td>
<td>187 (15.7)</td>
<td>2029 (16.3)</td>
</tr>
<tr>
<td>Holstein Friesian</td>
<td>169 (14.2)</td>
<td>871 (7)</td>
</tr>
<tr>
<td>Brown Swiss</td>
<td>156 (13.1)</td>
<td>1,605 (12.9)</td>
</tr>
<tr>
<td>Simmental</td>
<td>147 (12.3)</td>
<td>722 (5.8)</td>
</tr>
<tr>
<td>Other</td>
<td>281 (23.4)</td>
<td>5650 (45.4)</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural mating</td>
<td>1,123 (94.1)</td>
<td>Na</td>
</tr>
<tr>
<td>AI</td>
<td>71 (5.9)</td>
<td>Na</td>
</tr>
</tbody>
</table>

* Sampled bulls aged over 24 months.
** All bulls aged over 24 months living in Switzerland in 2012.

na: Data not available.
Scrapings from 1098 bulls’ penises, preputial washings from 61 naturally mating bulls, and 203 AI bulls, respectively, and abomasal fluids from 60 aborted fetuses were tested in parallel by in vitro culture and PCR. Samples from preputial washings and abomasal fluids were all negative by both methods. In the cultures of three scrapings from slaughtered bulls, motile trophozoites could be observed during all three days ($n=2$) or at one day ($n=1$), respectively, (Table 2) of cultivation. Subculturing in new InPouch™ cultures and in Diamonds medium (Diamond, 1957) failed. Panspecific PCR (Frey et al., 2009) of these three samples yielded amplification products of 378, 393 and 399 bp, respectively, which were larger than the 367 bp product expected for *T. foetus*. Subsequent cloning of the amplification products and DNA sequencing yielded *Tetra-trichomonas* sp. for all samples. The closest matches in GenBank as well as additional information on the bulls are shown in Table 2. Furthermore, PCR specific for *Tri-trichomonas* sp. (Felleisen et al., 1998) was performed for all three samples and remained negative. Based on these results, we concluded that all samples tested were negative for *T. foetus*. As in our study the system sensitivity to detect a prevalence of 0.002, 0.003 and 0.004 was 0.893, 0.967 and 0.990, respectively, we could conclude that the maximum prevalence that could have been missed was 0.3% (with 95% confidence).

4. Discussion

Natural mating is becoming increasingly popular in the Swiss cattle husbandry, but bulls used in this system are not routinely tested for venereal diseases. A re-introduction/re-occurrence of bovine *T. foetus* is conceivable either via resurgence from a yet unknown bovine reservoir (Mendoza-Ibarra et al., 2012), or via importation of infected cattle. The low disease awareness for bovine trichomoniasis might favor a hidden re-occurrence of *T. foetus* in the Swiss cattle population. The aim of our study was thus to search for the parasite, and if found, to assess the prevalence of bovine *T. foetus* in Switzerland.

In the present epidemiological survey, which is the first of this kind for Switzerland, we applied two sampling methods on bulls, namely preputial scrapings (from dead bulls) and preputial washings (from living bulls). To avoid death of the fragile trichomonads during their transport to the IPB, precautions were taken to avoid desiccation or thermal damage of the samples by sealing the penises/prepuces in plastic bags, and by shipping all samples at room temperature. However, to avoid false negative results in case of parasite death during transport, we did not solely rely on culture but performed PCR in parallel on each sample. The combination of culture and PCR on one sample had been shown to result in a diagnostic sensitivity of 78.3% (Cobo et al., 2007). Sensitivity could be optimized by repeated sampling respective to individual bulls (Cobo et al., 2007). In our study, maximal diagnostic sensitivity could not be achieved because most of our bulls could only be sampled once, namely upon slaughter. However, our sampling strategy had several advantages
that prompted us to tolerate this limitation: (1) the labor-intensive and potentially dangerous task of immobilizing the bull to take a preputial washing or scraping was not necessary, (2) a large number of bulls could be sampled within just one year, and (3) all post-mortem samples were processed by the same person under standardized laboratory conditions, thus reducing variations in diagnostic sensitivity due to changing handling of the samples. The detection of three bulls that harbored apathogenic Tetratrichomonas sp. indirectly confirmed the appropriateness of our methodological approach to diagnose and identify/specify trichomonad infections. These three samples were taken from dead bulls and both, culture and initial PCR, were positive for trichomonads. Previous reports reported findings of non-T. foetus trichomonads in preputial samples of routinely examined bulls and suggested that Tetratrichomonas sp. most likely represented contaminations from the digestive tract (Taylor et al., 1994; Cobo et al., 2003; Campero et al., 2003; Walker et al., 2003; Dufernez et al., 2007; Corbeil et al., 2008; Huby-Chilton et al., 2009).

The present survey unveiled two practices that seem to be widely used in herds using natural mating in Switzerland and that are both beneficial for the prevention of T. foetus infections: (1) the dominant use of young bulls in natural mating (Campero and Gottstein, 2007) as reflected by the age curve of both our sampled and the target population, and (2) the almost exclusive use of indigenous bulls, thus reducing the risk of importation of the disease. Furthermore, our study revealed that whole fetuses are only sporadically submitted for laboratory analyses. Thus, we could only test a limited number of abomasal samples that was insufficient to fulfill the statistical requirements of the present type of epidemiological survey.

Some concerns for cattle health had arisen after the detection of widespread infection of cats with feline T. foetus (Burgener et al., 2009; Frey et al., 2009), as cats on farms normally have unlimited access to barns and cattle, and as experimental transmission from cats to cattle is possible (Stockdale et al., 2007). However, recent studies unambiguously demonstrated that feline and bovine T. foetus exhibit conserved genetic differences (Reinmann et al., 2012; Slapeta et al., 2010, 2012; Sun et al., 2012), thus suggesting different host tropism (Slapeta et al., 2012).

5. Conclusions

Based on the results of this study, bulls used for natural mating in Switzerland have a probability of less than 0.3% of being infected with bovine T. foetus. However, we cannot exclude that the parasite might be present in some breeds and under particular forms of management, or that it might be re-introduced via infected imported animals. Therefore, despite of Switzerland being free of bovine trichomoniasis, disease awareness should be maintained in the future, and a statutory testing regimen for bulls imported from endemic areas should be considered.

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