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Research paper

Simplicimonas-like DNA in vaginal swabs of cows and heifers cross-reacting in the real-time PCR for *T. foetus*

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ABSTRACT

Cows on an alpine pasture were presented with severe signs of vaginitis. To rule out infection with *Tritrichomonas foetus*, vaginal swabs were taken and real-time PCR based on detection via fluorescence resonance energy transfer (FRET) probes and targeting the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA (rDNA) was performed. PCR was positive in 25 of totally 34 assessed cows. However, the melting profiles of the probes targeting the diagnostic PCR products differed from the *T. foetus* positive control. Subsequent sequencing of the amplicons revealed 91% identity to *Simplicimonas* sp. sequences deposited in GenBank™. Furthermore, there was no clear association between positive PCR result and presence of vaginitis. To investigate the distribution of this *Simplicimonas*-like organism in cows, more herds grazing on the same alpine pastures as well as unrelated cows were tested. In total, 133 cows and 16 heifers were sampled, 53 cows and 6 heifers even twice. Vaginitis was evident in 43 cows and 4 heifers. All-over-positivity of PCR was 44%, including nine tests performed on heifers. Melting peak analysis indicated *Simplicimonas*-like organisms in all positive samples. Culture attempts in bovine InPouch ™ TF failed. No association between a positive PCR result and the presence of vaginitis was found. This is, to the best of our knowledge, the first report on *Simplicimonas*-like DNA in vaginal swabs of female cattle. Our data suggest that when testing vaginal swabs of cattle by means of *T. foetus* PCR, false positive reactions due to *Simplicimonas*-like organisms may occur.

1. Introduction

Simplicimonas is a recently described parabasalid genus in the class Tritrichomonadea (Cepicka et al., 2010). The authors regrouped several species formerly known as *Monocercomonas* spp. to *Simplicimonas* and renamed them as *Simplicimonas* spp. Those were *S. moskowitzi* (isolated from reptiles), *S. cuniculi* (isolated from rabbits), *S. axostylis* (isolated from termites), *S. molae* (isolated from sunfish), *S. caviae* and *S. pistillum* (isolated from guinea pigs), and *S. lori* (isolated from slender loris) (Cepicka et al., 2010). A new species, *S. similis*, isolated from lined leave-tailed geckos, was also described (Cepicka et al., 2010). All those flagellates are regarded as commensals of the intestinal tract (Cepicka et al., 2010). Since the description of the genus *Simplicimonas*, isolates from the intestines of turkeys and chickens (Lollis et al., 2011; Bilic et al., 2014), rats (Kamaruddin et al., 2014), true bugs (Smejkalová et al., 2014), and water buffalo (Dimasuay et al., 2013) were referred to as *Simplicimonas* sp. Members of this young genus therefore seem to occur in various hosts. Up to now, no pathogenic effects have been attributed to those flagellates.

One other member of the class Tritrichomonadea, *Tritrichomonas foetus*, is a venereal pathogen in cattle responsible for substantial economic loss due to vaginitis and infertility in female cattle (BonDurant, 1997). Infection with *T. foetus* is notifiable (Terrestrial Animal Health Code, Chapter 11.13, Office Internationale des Epizooties O.I.E.) and to be eradicated in Switzerland (Anon., 1995) but has not been detected in cattle since 1997 in Switzerland (FSVO, 2016). Freedom from *T. foetus* in cattle was also confirmed in a recent study targeting animals at risk (Bernasconi et al., 2014). As infected bulls are asymptomatic carriers of *T. foetus* and may thus spread the parasite, control measures mainly rely on detecting such infected bulls via cultivation methods or PCR assays (BonDurant,

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1997; Felleisen, 1997). Standard diagnostic methods are applied according to the O.I.E. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 2.4.16 Trichomonosis (OIE, 2012). Real-time PCRs using specific probes for detection of *T. foetus* were considered as highly specific methods (McMillen and Lew, 2006). However, false-positive PCR-results due to *Simplicimonas* spp. were observed in samples obtained from bulls (Schommer et al., 2011). In contrast, a further multicentre study using a real-time PCR for the detection of *T. foetus*, tested a total of 803 bull smegma samples to assess potential cross-reactions, but none were detected (Effinger et al., 2014).

In September 2016, severe cases of vaginitis and vaginal discharge were observed in a herd of cows and heifers on a Swiss alpine pasture. As endemic cases of vaginitis are rarely seen in Switzerland, the private practitioner wanted to rule out infection with *T. foetus* and sent vaginal swabs to the Swiss reference laboratory. Our data suggest that when testing vaginal swabs of cattle by means of *T. foetus* PCR, false positive reactions due to *Simplicimonas*-like organisms may occur.

2. Materials and methods

2.1. Cows and heifers: case history

In September 2016 in a herd (A) of total 34 cows on an alpine pasture n = 29 cows were found with severe signs of vaginitis, *i.e.* redness, disseminated vesicles (diameter 1–2 mm), few pustules, and intermittent vaginal discharge (Fig. 1a,b and Table 1). Blood testing was negative for BVDV and BHV1 in all cows. Swabs from the vagina revealed normal vaginal flora, PCR analysis for Chlamydiales and Mycoplasma was negative. First sampling was done by the private practitioner responsible for the alpine pasture. All subsequent samplings were done by GH and CF. Thereafter, contact herds (B & C) on the alpine pasture considered as healthy by the herdsmen were visited. However, 15 out of 67 cows and heifers showed mild signs of vaginitis (Table 1). Additionally, 5 healthy cows (D) in the village below the alpine pasture were sampled (Table 1). As unrelated control, a herd near Bern (E), with animals never staying on alpine summer pastures, undergoing routine fertility visits once a fortnight consisting of n = 26 cows and n = 7 heifers was sampled. In 2 cows, a slight vaginitis (without pustules) was present (Table 1). In none of the herds a bull was present, and all herds relied on artificial insemination for breeding.

In herd (A), a second sampling was done already after two weeks (Table 1). Herds (C) and (D) were revisited and sampled three months after the initial visit (Table 1). Herds (B) and (E) were only sampled once. In total, 133 cows (43 of them with vaginitis) were sampled. 80 cows (11 with vaginitis) were sampled once, 53 cows (32 with vaginitis) were sampled twice. In total, 16 heifers (4 with vaginitis) were sampled. 10 heifers (1 with vaginitis) were sampled once, 6 heifers (3 with vaginitis) were sampled twice.

2.2. Vaginal swabs

Perineum and vulva of the animals were dry-cleaned with paper. Vaginal cotton swabs were taken out of the vagina (vulvar labia spread) from each cow or heifer and transferred to physiological saline (0.9% NaCl) or directly incubated in bovine InPouch™ TF media (Biomed, White City, Oregon).

2.3. DNA extraction

Tubes containing 0.9% NaCl and cotton swabs were centrifuged at 300 × *g* for 5 min. Sediments were collected and 100 μl used for DNA extraction and 200 μl for inoculation of InPouch™ TF media. Swabs directly inoculated in InPouch™ TF: 100 μl of sediment was obtained from InPouch™ TF using glass Pasteur pipettes. DNA was extracted using Qiagen DNA-extraction kit (Qiagen, Germany) following the manufacturer's protocol for cultured cells. DNA was eluted in 200 μl elution buffer. 1 μl of DNA was used in the PCR reactions.

2.4. Real-time PCR

Diagnostic real-time PCR targeted the 5.8S rDNA and flanking ITS-1 and ITS-2 regions of *T. foetus*. Fluorescence resonance energy transfer (FRET) probe-based real-time PCR was done using published primers forward 18S primer: 5′-GTAGGTGAACCTGCCGTTG-3′ and reverse 5.8S primer: 5′-TTCAGTTCAGCGGGTCTTC-3′ (MWG-Biotech Inc., Germany) (Frey et al., 2009), together with probes 3′-fluorescein labelled ITS1-3FL: 5'-GTTGCATAATGCGATAAGCGGCT-3' and 5′-LC-Red 640 labelled ITS1-5LC: 5'-GATTAGCTTTCTTTGCGACAAGTTCGAT-3' (TIB MOLBIOL, Munich, Germany). Real-time PCR was run on a Light Cycler™ 2.0 instrument (Roche Diagnostics, Basel, Switzerland). In each run, a positive control containing 1 μl of *T. foetus* DNA (ATCC 30924) and a negative control containing 1 μl of sterile water was included. Reactions of 10 μl contained 1 μl of 10× LightCycler DNA Master Hybridisation Probes™ Kit (Roche Diagnostics), containing dUTP instead of dTTP. Each PCR mix contained 0.5 μM of each of the *Tritrichomonas* sp.-specific primers, 0.3 μM of each probe (MWG-Biotech Inc., Germany), and 0.125 units of uracil DNA glycosylase (UDG) (Roche Diagnostics). $MgCl_2$ was supplemented to a final concentration of 3 μM. UDG and dUTP were included in the reaction mixture to prevent carry-over contamination (Longo et al., 1990). The samples were incubated for 30 min at 40 °C prior to the PCR reaction for UDG-mediated decontamination, followed by a 15 min denaturation of the DNA at 95 °C. Subsequent DNA amplification was done in 50 cycles (denaturation [94 °C, 10 s], annealing [60 °C–53 °C, touch-down 1 °C per cycle, 20 s], and extension [72 °C, 30 s]). After the last cycle, a melting curve was effectuated by continuously increasing temperature from 50 °C to 95 °C and measuring loss of fluorescence signal using corresponding standard software (v.3.5.3) (Roche Diagnostics).

To detect inhibitory effects of the sample DNA, a parallel reaction was carried out for each PCR reaction containing sample DNA plus 1 μl of positive control DNA.

Samples showing positive reactions were re-amplified in conventional PCR according to Frey et al. (2009) and amplification products were sent to a commercial sequencing service (Microsynth, Balgach, Switzerland). The sequence was deposited in GenBank™ under the accession number KY410341.

ClustalW sequence alignments were done using the open source software MUSCLE (3.8) (http://www.ebi.ac.uk/Tools/msa/muscle/).

2.5. Culture

If vaginal swabs were not directly inoculated in bovine InPouch™ TF media, approximately 200 μl of sediment of vaginal swabs in physiological saline was inoculated in a pouch. InPouch™ TF were vertically incubated at 37 °C for 11 days. They were microscopically checked for growth of protozoans every 24 h for the first three days, then every 48 h. In total, 141 samples were incubated in InPouch™ TF.

2.6. Statistics

Association between vaginitis and PCR-result was assessed by the two-tailed Fisher's exact test (Agresti, 2002) with the R-software (version 3.3.1, R Core Team, 2016). The level of significance was set at $p < 0.05$.

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