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Short communication

Diagnosis of *Sarcocystis* spp. in cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam

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ABSTRACT

Our aim was to develop a method for species diagnosis and to obtain data on the prevalence of *Sarcocystis* infections in cattle and water buffalo in the Son La Province of Northern Vietnam. Meat samples of naturally infected animals were examined by light and electron microscopy as well as by molecular methods. A PCR of part of the 18S rDNA gene followed by RFLP analysis was modified to detect infections with different *Sarcocystis* spp. in cattle and water buffaloes slaughtered in the Son La Province. It showed to be an economical method to detect multiple infections with *Sarcocystis* spp. Sequence analysis of the PCR amplicons was performed with selected samples and the results were compared with published sequences. With these methods the following *Sarcocystis* spp. were identified in cattle: *Sarcocystis hirsuta*, *Sarcocystis cruzi* and *Sarcocystis hominis*.

Water buffaloes were infected with *Sarcocystis fusiformis*, *S. cruzi*, *S. hominis* and *S. hirsuta*. The results indicate that *Sarcocystis* spp. infecting cattle are also able to infect water buffaloes. So the validity of certain *Sarcocystis* spp. of water buffalo is discussed. Bovine lifestock in Northern Vietnam were commonly infected with *Sarcocystis* spp.

1. Introduction

Sarcocystis spp. are cyst-forming intracellular protozoan parasites with an obligate two host life cycle between predators as final hosts and their prey animals as intermediate hosts. *Sarcocystis* spp. are highly prevalent in livestock animals and are considered to be very host specific. Therefore, it is assumed that water buffaloes as well as cattle are solely infected with their own species. Cattle are mainly infected with *Sarcocystis cruzi*, *Sarcocystis hominis* and *Sarcocystis hirsuta* (Tenter, 1995; Dubey and Lindsay, 2006). Water buffaloes are intermediate hosts for *Sarcocystis fusiformis*, *Sarcocystis levinei* (*S. cruzi*-like species), *Sarcocystis dubeyi*, *Sarcocystis sinensis* (both *S. hominis*-like species) and *Sarcocystis buffalonis* (*S. hirsuta*-like species) (Dubey et al., 1989b; Huong et al., 1997a,b; Huong, 1999; Yang et al., 2001a,b). The *Sarcocystis* spp. can be differentiated due to their specific cyst wall structure (survey see Dubey et al., 1989a,b,c; Tenter, 1995) and by molecular methods as well as by sequence analysis (Tenter et al., 1994; Heckeroth and Tenter, 1999).

Few publications exist concerning the prevalences of *Sarcocystis* spp. in water buffaloes (Huong et al., 1997a,b; Huong, 1999) and similar data on prevalences in cattle from Northern Vietnam are lacking.

The aim of our project was to obtain data on the prevalence in these livestock animals.

We established a rapid method to distinguish between the different *Sarcocystis* spp. *S. cruzi*, *S. hominis* and *S. hirsuta* infecting cattle. PCR-RFLP analysis with different restriction endonucleases showed a unique fragment pattern for each of these species, so they can be clearly

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identified in a mixture of different *Sarcocystis* DNA. The results were confirmed by sequence analysis of the 18S rRNA gene.

To identify the different *Sarcocystis* spp. infecting water buffalo we based our method on the findings of Yang et al. (2001a,b, 2002) and Li et al. (2002). They compared *Sarcocystis* cysts (*S. cruzi*, *S. hominis* and *S. hirsuta*) from cattle and the corresponding cysts from water buffalo morphologically, by sequence analysis of the 18S rRNA gene and by PCR-RFLP. They combined the results and recognized that the *Sarcocystis* cysts from both cattle and water buffalo can be considered the same species (i.e. *S. cruzi*, *S. hominis* and *S. hirsuta*) and that water buffalo serves as their intermediate host in nature. Therefore, we used the same restriction endonucleases with samples of *Sarcocystis* DNA obtained from water buffalo and beneath the identification of *S. fusiformis* they showed identical fragment patterns with the samples obtained from cattle. These findings were supported by the results of sequence analysis of the 18S rRNA gene, showing an up to 100% identity with *Sarcocystis* spp. of cattle. Under these circumstances in the following the species names *S. cruzi*, *S. hominis* and *S. hirsuta* are used for *Sarcocystis* spp. infecting water buffalo, replacing the names *S. levinei*, *S. dubeyi* and *S. buffalonis*.

It was not our aim to identify *Sarcocystis* spp. using experimental infections but to find an easy method for species diagnosis via PCR-RFLP in naturally infected animals.

This project was part of the SFB (Sonderforschungsbereich) "Uplands Program – Research for sustainable land use and rural development in mountainous regions of Southeast Asia" funded by the Deutsche Forschungsgemeinschaft. Therefore, the research was restricted to rural areas in Northern Vietnam.

2. Materials and methods

The survey was done from October until December 2003 in 30 abattoirs in the town of Son La in the Son La Province in Northern Vietnam, where meat inspection is only performed sporadically and dogs and cats have often access to meat and offal disposals.

Meat inspection was done on 101 cattle and 30 water buffaloes, which originated from various small villages in Son La Province. From each animal tissue samples (each 30–50 g) from tongue, cervical muscle, oesophagus and diaphragm were collected during meat inspection. In the laboratory 0.5 mm sized pieces of the samples were squashed between two glass slides and inspected under a stereomicroscope (16× magnification) for microscopically visible *Sarcocystis* cysts. In total 541 samples of cattle and 208 samples of water buffaloes were inspected microscopically. If cysts were found in the sample, several 1 cm long pieces of tissue were fixed either in 70% ethanol or 10% neutral buffered formalin, the obtained macroscopically visible cysts were handled the same way. In total, 945 samples from cattle and 478 samples of water buffaloes were fixed for further studies. For light microscopy pieces of the formalin fixed samples were processed by standard histological techniques, sectioned at 7 μm, stained with Haematoxylin and Eosin and examined at 400–1000× magnification for *Sarcocystis* cysts.

For electron microscopy small pieces of tissue (max. 2 mm × 5 mm) containing many cysts and large single cysts were fixed in 2.5% glutaraldehyde in 0.1 M Soerensen buffer pH 7.4 and stored at 4 °C. The samples were post-fixed with 1% osmiumtetroxide and blockstained with 6% uranylacetate, dehydrated in graded ethanol and embedded in araldite. Ultrathin sections were stained with lead citrate and studied electron microscopically (LEO 912 AB).

DNA was isolated from all ethanol preserved samples positive for *Sarcocystis* according to Dinkel et al. (2004): 0.1–0.3 g of the samples were cut into small pieces and digested in the presence of 2 mg/ml proteinase K in 500 μl of 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulphate and 20 mM dithiothreitol. DNA was extracted using phenol–chloroform extraction and subsequent ethanol precipitation (Sambrook et al., 1989). After drying, DNA was suspended in 100 μl nuclease-free water and its concentration measured photometrically.

For PCR the target sequence chosen for amplification was part of the mitochondrial 18S rRNA gene. Those variable regions have shown to be suitable genetic markers for distinguishing *Sarcocystis* spp. (Yang et al., 2002). Primers were used according to Li et al. (2002), the forward primer 18S9L (5′ GGA TAA CCT GGT AAT TCT ATG 3′) and the reverse primer 18S1H (5′ GGC AAA TGC TTT CGC AGT AG 3′) amplifying a fragment of approx. 900 bp. The used primer combination is *Sarcocystis* genus-specific and does not amplify host DNA (Li et al., 2002).

PCR was performed in a total volume of 100 μl using 400 ng DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, each desoxynucleoside triphosphate at a concentration of 200 μM, 60 pmol of each primer and 2.5 units Ampli-Taq Polymerase (Applied Biosystems) as follows: 94 °C for 3 min, followed by 40 cycles of 94 °C for 40 s, 56 °C for 60 s, 72 °C for 80 s and finally 72 °C for 5 min.

Amplification products as well as a negative control were separated on a 1.5% agarose gel stained with ethidium bromide (TBE-buffer, 55 V, 45 min).

For PCR-RFLP analysis the available sequences of the 18S rDNA gene of the different *Sarcocystis* spp. were extracted from GeneBank. The sequences were compared and restriction sites were identified using the Harvard program (http://pga.mgh.harvard.edu/web_apps/web_map/start) in order to differentiate between different *Sarcocystis* spp. as tissue samples may have contained cysts of more than one *Sarcocystis* sp. Therefore, banding patterns generated by PCR-RFLP include the individual fragment patterns each of which is characteristic for one species indicating multiple infections. The accession numbers, the chosen restriction endonucleases for RFLP and the species specific fragment sizes are listed in Table 1. Amplified PCR products were digested separately with restriction enzymes. A total of 50 μl reaction mixture was used containing 10–20 μl PCR product, 10 units restriction enzyme and 5 μl appropriate buffer. The restriction mixture was incubated for 16 h at 37 °C (Dra1, Ssp1) or 55 °C (Fok1, Bsl1). Enzymes were inactivated for 20 min at