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Design and validation of an oligonucleotide probe for the detection of protozoa from the order Trichomonadida using chromogenic in situ hybridization

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ABSTRACT

Infections with protozoal parasites of the order Trichomonadida are often observed in veterinary medicine. Based on the trichomonad species involved these infections are either asymptomatic or can lead to sometimes serious disease. To further study protozoal agents of the order Trichomonadida the establishment of a method to detect trichomonads directly in the tissue, allowing parasite–lesion correlation, is necessary. Here we describe the design and evaluation of an oligonucleotide probe for chromogenic in situ hybridization, theoretically allowing detection of all hitherto known members of the order Trichomonadida. The probe was designed on a region of the 18S ribosomal RNA gene homologue for all representatives of the order Trichomonadida available in the GenBank. Functionality of the probe was proven using protozoal cultures containing different trichomonads (*Monocercomonas colubrorum*, *Hypotrichomonas acosta*, *Pentatrichomonas hominis*, *Trichomitus batrachorum*, *Trichomonas gallinae*, *Tetratrichomonas gallinarum*, *Tritrichomonas foetus*, and *Tritrichomonas augusta*). Furthermore, three different tissue sections containing either *T. gallinae*, *T. foetus* or *Histomonas meleagridis* were tested positive. Additionally, to rule out cross-reactivity of the probe a large number of different pathogenic protozoal agents, fungi, bacteria and viruses were tested and gave negative results. The probe presented here can be considered an important tool for diagnosis of all to date described relevant protozoal parasites of the order Trichomonadida in tissue samples.

1. Introduction

The order Trichomonadida belongs to the phylum Parabasala and comprises anaerobic and amitochondrial protists. The order is further subdivided into four families—Monocercomonadidae, Trichomonadidae, Tritrichomonadidae and Trichomitidae (Hampl et al., 2006, 2007). All trichomonads harbor hydrogenosomes instead of mitochondria and possess up to six flagella. Aside from the Monocercomonadidae all protozoa from this order exhibit an undulating membrane (a motility organelle) and costa or its rudiment (most likely for mechanical support).

Most trichomonads live as symbionts or parasites in the intestine, but there are also several free living species. The order Trichomonadida includes human and veterinary

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pathogenic species. *Trichomonas vaginalis* is the most common and best studied trichomonad in human beings and causes trichomonosis, a sexually transmitted infection of the urogenital tract. In veterinary medicine *Tritrichomonas foetus*, *Trichomonas gallinae* and *Histomonas meleagridis* are of high importance. *T. foetus* is the causative agent of bovine trichomonosis (Parsonson et al., 1976), a sexually transmitted disease leading to uterine infections, abortions and sterility in heifers. The same trichomonad causes colitis in cats (Levy et al., 2003) and can also be found in the colon of pigs (Tachezy et al., 2002). *T. gallinae* causes avian trichomonosis and can be detected in the oral cavity, pharynx, esophagus and crop of birds especially of the order Columbiformes (Stabler, 1954). Another parasite found in many gallinaceous birds is *H. meleagridis* (Tyzzer, 1920) causing a typhlohepatitis known as histomonosis or blackhead disease. Besides these well characterized protozoal agents, there still exist many not well studied trichomonad species. Furthermore, trichomonad infections often do not cause obvious lesions or trichomonads are overlooked in histological slides after hematoxylin and eosin (HE) staining.

Common methods to detect different trichomonad species either involve wet mount preparations followed by cultivation and microscopical examination (Mehlhorn et al., 2009) or PCR analysis and nucleotide sequencing (Gookin et al., 2007). These methods, however, do not provide information concerning pathogenicity of the protozoal agents. Thus, a technique to visualize the organisms directly in tissue sections is desirable. Chromogenic in situ hybridization (ISH) proved to be a robust and reliable method, which enables detection of the respective parasite directly in tissue, in many cases in association with induced lesions (Chvala et al., 2006). This technique had already been successfully applied by Richter et al. (2008) for detection of *Monocercomonas* sp. and by Liebhart et al. (2006) for three different protozoal agents of the order Trichomonadida. To further study protozoa from this order, an ISH probe with the ability to virtually detect all hitherto known members of the order Trichomonadida (OT probe) seemed to be a useful tool. This probe would allow quick screening of samples, assessment of pathogenicity of the found trichomonads as well as detection of unknown trichomonad species. Here we describe the design and extensive validation of such a probe and its use in chromogenic ISH.

2. Materials and methods

2.1. Probe design

An ISH probe for the detection of all protozoal agents of the order Trichomonadida was designed: order Trichomonadida (OT) probe. First, extensive homology studies using the Sci Ed Central (Scientific & Educational software, Cary, NC, USA) software package were carried out on all available GenBank sequences of the 18S ribosomal RNA (rRNA) gene of protozoa from the order Trichomonadida. A region of strong homology (with a maximum of two nucleotides difference) present in 18S rRNA gene sequences of all trichomonads was chosen as probe sequence. The selected OT probe sequence was 5′-TTG CGG TCG TAG TTC CCC CAG AGC CCA AGA ACT-3′. Subsequently, this sequence was submitted to Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast.cgi) to search against GenBank sequences and exclude unintentional cross-reactivity. The OT probe sequence was sent to Eurofins MWG Operon (Ebersberg, Germany) for probe synthesis and labeling of the 3′ end with digoxigenin. Consequently, ISH was carried out using the newly designed probe on different protozoal cultures and tissue samples.

2.2. In situ hybridization

Chromogenic ISH was performed according to a previously described protocol (Chvala et al., 2006). Briefly, 3 μm paraffin sections were dewaxed and rehydrated. For proteolysis the slides were treated with proteinase K (Roche, Basel, Switzerland) 2.5 μg/ml in Tris-buffered saline for 30 min at 37 °C. Afterwards the slides were rinsed in distilled water, dehydrated in alcohol (95% and 100%) and air-dried. The slides were incubated overnight at 40 °C with a hybridization mixture, 100 μl of which were composed of 50 μl formamide, 20 μl 20× standard sodium citrate (SSC), 10 μl dextran sulfate (50%, w/v), 12 μl distilled water, 5 μl boiled herring sperm DNA (50 mg/ml), 2 μl 50× Denhardt's solution and 1 μl OT probe at a concentration of 20 ng/ml. On the second day the slides were washed with decreasing concentrations of SSC (2× SSC, 1× SSC, 0.1× SSC; 10 min each) to remove non-hybridized probe. Afterwards the slides were incubated with anti-digoxigenin-AP Fab fragments (Roche) (1:200) for 1 h at room temperature. Visualization of the hybridized probe was carried out after an additional washing step using the color substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche). Color development was stopped with TE buffer (pH 8.0) after 1 h of incubation. The slides were counterstained with hematoxylin and mounted under coverslips using Aquatex (VWR International, Vienna, Austria).

2.3. Positive and negative control samples

Several protozoal cultures embedded in paraffin wax, displaying representatives of the order Trichomonadida, were used as positive controls for the designed ISH probe. Cultures contained *Monocercomonas colubrorum* (Richter et al., 2008), *Hypotrichomonas acosta*, *Pentatrichomonas hominis* (GenBank accession no. AY349187) (Kleina et al., 2004), *Trichomitus batrachorum*, *T. gallinae*, *Tetratrichomonas gallinarum* (Liebhart et al., 2006), *T. foetus* (Tachezy et al., 2002), and *Tritrichomonas augusta* (GenBank accession no. AY055802) (Tachezy et al., 2002) (culture collection of the Department of Parasitology, Faculty of Science, Charles University Prague and of the Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Vienna). All protozoal cultures containing an unknown number of parasites were fixed in 7% buffered formalin and embedded in paraffin wax. Prior to embedding all cultures were soaked with rice starch (3.3 mg/ml) for 5 h and centrifuged at 6000 × *g* for 10 min to produce a pellet. The pellet was overlaid with agar, hardened at 4 °C, carefully