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APPLICATION OF SPECIFIC PRIMERS IN THE DIAGNOSIS OF *ENCEPHALITOZOON* SPP.

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Abstract: In our experiment, 3 species-specific primer pairs cultivated in cell lines were used: *Encephalitozoon cuniculi*-specific primer pairs (ECUNF and ECUNR), *Encephalitozoon hellem*-specific primer pairs (EHELF and EHELR), and *Encephalitozoon intestinalis*-specific primer pairs (SINTF and SINTR). The PCR products were estimated to be 550 bp in *E. cuniculi*, 547 bp in *E. hellem* and 545 bp in *E. intestinalis* respectively, which can prove the precision and reliability of this method in the species identification of the genus *Encephalitozoon*. All 3 primer pairs were species-specific and none of them amplified gene sequences from other *Encephalitozoon* spp.

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INTRODUCTION

Encephalitozoonis is a microsporidian infection caused by obligate intracellular parasites of the genus *Encephalitozoon* that are a serious cause of disease, especially in HIV-positive patients. However, more and more frequently, these are pathogenic to other patients with immunosuppression of different aetiology. So far, over 100 cases of infections with *Encephalitozoon* spp. have been detected in humans. Although the majority of these were caused by *E. intestinalis* or *E. hellem* [1, 4], *E. cuniculi* was recently identified in some HIV-infected patients [6].

Therefore, methods for the diagnosis and species differentiation of microsporidia should be available in every laboratory that performs parasitological examinations of clinical samples.

Lately, several methods based on the polymerase chain reaction (PCR) for the amplification of different regions of the SSU and LSU rRNA genes have been described, as well as the intergenic spacer region for the diagnosis and species differentiation of microsporidia infecting humans and animals [3]. This method appears to be relatively quick and highly precise in the detection of the parasite when making use of species-specific primer pairs for the amplification of specific DNA sequences.

The goal of the work performed was to verify the suitability of the application of specific primer pairs ECUNF and ECUNR, SINTF and SINTR, as well as EHELR and EHELF for *E. cuniculi*, *E. intestinalis* and *E. hellem*, respectively, with the purpose of their diagnosis in infected individuals.

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MATERIALS AND METHODS

Spores of *E. cuniculi*, *E. hellem* and *E. intestinalis* obtained from the infected RK13 cell line (cultivated and used as an antigen in our laboratory), cultivated in the RPMI 1640 medium supplemented with 5% BOFES (bovine foetal serum) and with addition of antibiotics and antimycotics. Fresh spores were isolated from the medium [8] and used in a PCR.

Primers. We used 3 specific primers pairs ECUNF (*180N2: 5‘- ATG AGA AGT GAT GTG TGT GCG -3‘) : ECUNR (*180N3: 5‘- TGC CAT GCA CTC ACA GGC ATC - 3‘), SINTF (*180N4: 5‘ - TAT GAG AAG TGA GTT TTT TTC - 3‘): SINTR (*180N5: 5‘ - CCG TCC TCG TTC TCC TGC CCG - 3‘), EHELF (*216C5: 5‘ - TGA GAA GTA AGA TGT TTA GCA - 3‘): EHELR (*216C6: 5‘ - GTA AAA ACA CTC TCA CAC TCA - 3‘; Generi Biotech, Czech Republic) with the concentration of 25 pmol.μl^{-1}.

DNA extraction and PCR amplification. Suspension of spores in PBS (100 ml) was subjected to mechanical microwave disruption 3 × 20 sec. For the DNA extraction, tissue lyses buffer containing SDS (Sodium Dodecyl Sulfate, JETquick Tissue DNA Spin Kit, Genomed, USA) and 25 µl proteinase K (25 mg.ml^{-1}) were used. The specimen was incubated for 1 hour at 56ºC and further processed in compliance with the DNA Spin Kit Manufacturer’s Instructions. In the last step of DNA purification, water was used in the PCR.

Per 1 reaction, the PCR set contained 2.5 µl TAQ puffer adjusted to 3 mM with $MgCL_2$, 1.0 µl dNTPs (0,2 mM), 0.5 µl TAQ purple polymerase, 1.0 µl 25 pmol solution of each primer used to reaction, 2.0 µl DNA template isolated from *E. cuniculi*, *E. intestinalis* and *E. hellem* respectively, and water for the PCR ad 25.0 µl. (Oborník, personal announcement).

RESULTS

In our experiment, 3 species-specific primer pairs were used: ECUNF and ECUNR, EHELF and EHELR, and SINTF and SINTR for the amplification and identification of *E. cuniculi*, *E. hellem* and *E. intestinalis* respectively. The sizes of fragments of the amplified products were compared to the standard 100 bp DNA Ladder, which contained fragments of known size.

In the electrophoresis, the PCR products were characterised to be 550 bp in *E. cuniculi*, 545 bp in *E. intestinalis* and 547 bp in *E. hellem* respectively, which provided evidence for the precision and reliability of this method in the identification of *Encephalitozoon* spp. All 3 primer pairs were species-specific and none of them amplified gene sequences of other *Encephalitozoon* spp.

DISCUSSION

The first sequence data of SSU-rRNA of microsporidia were reported by Vossbrinck *et al.* approximately in 1987 [10]. Subsequently, PCR amplification with primers complementary to conserved sequences of the *V. necatrix* SSU rRNA gene was used to amplify and sequence DNA fragments of the small ribosomal subunits (SSU) rRNA gene from other mammalian micrisporidia [5, 9]. Nowadays, several nucleotide sequences of SSU and LSU rRNA genes from various other microsporidian species are known, including *Encephalitozoon* spp., such as *E. cuniculi*, *E. hellem*, and *E. intestinalis*. All these are accessible via the GenBank and EMBL databases.

The *E. cuniculi* SSU rRNA sequence was first described by Zhu *et al.* (1993) and the data obtained were deposited in the GenBank database under Accession No. Z19563 [12]. The authors performed the amplification of the SSU-rRNA gene with the universal primer pair V1 and 1492 from rabbit kidney cells (RK13) infected *in vitro* conditions with the microsporidia of *E. cuniculi* and *E. intestinalis*. This primer pair amplifies gene sequences of many microsporidian species and in the majority of them generates a 1,200–1,500 bp product.

In phylogenetic studies, a universal primer pair (530:580) was used for the amplification of the ITS region, portions of the SSU rRNA gene and portions of the LSU rRNA gene from a number of microsporidia [11, 12]. These universal primer pairs are useful for obtaining the sequence data of rRNA genes of other microsporidia; nevertheless they are not suitable for diagnosis, as the great size of their amplicon restricts their sensitivity.

In 1994, Visvesvara *et al.* [9] designed 2 species-specific primer pairs for both *E. cuniculi* (positions 344 to 364 and 872 to 892 of the *E. cuniculi* SSU rRNA sequence [ECUN-F and ECUN-R]) and *E. hellem* (positions 358 to 378 and 884 to 904 of the *E. hellem* SSU rRNA sequence [EHEL-F and EHEL-R]) for species-specific amplification of DNA fragments. These 2 primer pairs were used also for species differentiation of cultured organisms and organisms present in various clinical samples [6, 7].

Primer pairs SINTF and SINTR, described by Da Silva *et al.* [2], amplify cloned *E. intestinalis* SSU rRNA sequences and *E. intestinalis* DNA from duodenal-jejunal segments from patients with AIDS and suspected intestinal microsporidiosis, and from 8 different isolates from cell cultures. Specificity was tested with microsporidian SSU rRNA coding regions of *E. cuniculi*, *E. hellem*, *E. intestinalis*, E. bieneusi and *V. corneae*. Positive amplification was shown only with the *E. intestinalis* SSU rRNA. PCR products generated with these primer pairs were analysed by ethidium bromide-stained gel analysis and were identified by Southern blot hybridisation, DNA sequencing and restriction enzyme digestion.

To date, only limited data have been published comparing molecular methods for diagnosis of

microsporidia with traditional methods of determining the sensitivity and specificity of these new techniques. Despite that, these known studies indicate that the sensitivity and specificity of molecular methods is probably very high. Comparative traditional light-microscopic examination methods and PCR-based methods will still remain necessary in the future. For the detection of microsporidia in clinical samples, chemofluorescent or chromotrope-based staining techniques should be used first and foremost. After the identification of microsporidia by light microscopy, molecular methods should be applied for the confirmation of the presence of microsporidia and their species differentiation.

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