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# Sensitivity, specificity and comparison of three commercially available immunological tests in the diagnosis of *Cryptosporidium* species in animals

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ABSTRACT

The study was conducted to compare the specificity of immunological diagnostic methods used for the diagnosis of *Cryptosporidium* species capable of causing life-threatening infection in both immunosuppressed and immunocompetent patients. For the detection of *Cryptosporidium* species in 79 animals with diarrhoea, we used three Copro-antigen tests: RIDASCREEN® *Cryptosporidium* test, *Cryptosporidium* 2nd Generation (ELISA) and RIDA® QUICK *Cryptosporidium*. For immunoassays we used positive and negative samples detected by means of polymerase chain reaction and validated by sequencing and nested polymerase chain reaction to confirm the presence six different species of *Cryptosporidium* species. Prevalence of cryptosporidiosis in the entire group determined by enzyme immunoassay, enzyme linked immunosorbent assay, immuno-chromatographic test and polymerase chain reaction was 34.17%, 27.84%, 6.33% and 27.84%, respectively. Sensitivity of animal samples with enzyme immunoassay, enzyme linked immunosorbent assay, and immuno-chromatographic test was 63.6%, 40.9% and 22.7%, resp., when questionable samples were considered positive, whereas specificity of enzyme immunoassay, enzyme linked immunosorbent assay and immuno-chromatographic test was 75.9%, 78.9% and 100%, respectively. Positive predictive values and negative predictive values were different for all the tests. These differences results are controversial and therefore reliability and reproducibility of immunoassays as the only diagnostic method is questionable. The use of various *Cryptosporidium* species in diagnosis based on immunological testing and different results obtained by individual tests indicate potential differences in Copro-antigens produced by individual *Cryptosporidium* species.



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## Introduction

Cryptosporidia are cosmopolitan widespread parasite, with broad host specificity, primarily occurring in young livestock. Humans are also susceptible, especially immunodeficient individuals. In the recent years, considerable attention has been paid to cryptosporidiosis caused by zoonotic species, especially to host specificity of these species, and the associated possibility of disease transmission between different hosts in the environment.

Clinical manifestation of cryptosporidiosis comprises asymptomatic forms but also severe chronic states causing damage to the gastrointestinal tract and accompanied by diarrhoea, anorexia, cachexia, dehydration with dissemination of parasites to the surrounding organs with potential fatal impact on immunosuppressed subjects.[1,2] Cryptosporidiosis with clinical manifestation as well as asymptomatic shedding of oocysts is more frequent in the young than in adults. Reliable and early diagnosis is required not only of infection with fatal consequences but also of asymptomatic infections.[3,4]

A direct microscopic diagnosis of *Cryptosporidium* from stool samples is laborious and requires qualified personnel to identify the pathogen. The diagnostic accuracy is significantly reduced by a low concentration of oocysts or by mechanically/enzymatically damaged oocysts and irregularly excreted oocysts.[5,6] Worldwide seroprevalence in livestock reaches 27–30%.[7]

In the recent years, Copro-antigen commercial tests, such as enzyme immunoassay (EIA), or immunochromatic dipstick test (ICT) have been used for rapid diagnosis. According to the manufacturer, these tests are rapid and sensitive enough, but provide only quantitative results which suffice only for detection of the presence of pathogen in the holdings but not for individual diagnosis and identification.[6,8] Diagnosis based on Copro-antigen as a single test for detecting the presence of cryptosporidia is inadequate, particularly in risk groups such as immunodeficient patients with life-threatening diarrhoea.[9] Therefore, molecular methods, including polymerase chain reaction (PCR), became reference methods for the detection, identification, differentiation and generic genotyping of *Cryptosporidium* spp.[10,11]

The aim of our study was to evaluate and compare three commercially available Copro-antigen tests, namely RIDASCREEN® *Cryptosporidium* test (Enzyme Immunoassay – EIA), *Cryptosporidium* 2nd Generation (Enzyme Linked Immunosorbent Assay – ELISA), and RIDA®QUICK *Cryptosporidium* (Immuno-chromatographic test – ICT). PCR method, sequencing and phylogenetic analysis were used to confirm the presence of cryptosporidia and disprove false positivity and negativity of samples.

## Materials and methods

### *Study population – samples*

Stool samples were collected from 79 animals divided into three groups (1st – 35 pigs; 2nd – 34 calves; 3rd – 10 lambs), with clinical symptoms (diarrhoea, abdominal pain, anorexia, weight loss, dehydration).

By means of PCR analysis and subsequent sequencing, we truly detected positive and negative samples that were used for the immunoassay. We used 22 positive samples of varying localization. Intestinal species: calves – *C. parvum* (10); *C. bovis* (2); pigs – *C. scrofarum* (5), *C. suis* (2); and gastric species: pigs – *C. muris* (2), *C. andersoni* (1). The sensitivities, specificities, positive predictive values and negative predictive values were calculated according to Loong.[12]

### *Molecular analysis*

#### *DNA isolation*

Genomic DNA was extracted from 100 mg of stool sample using a DNA-Sorb-B Nucleic acid Extraction kit (AmpliSence, Russia) according to the manufacturer's instructions. Before extraction, we homogenized the stool and disrupted oocysts at 6500 rpm for 90 s with addition of 0.5-mm-glass beads, 1.0-mm-zircon beads and 300 μL lysis solution in a homogenizer Precellys 24 (Bertin technologies). Purified DNA was stored at −20 °C until use in PCR.

#### *Nested PCR*

Using a modified protocol for nested PCR we amplified 350 bp long amplicons specific for 18SSU r RNA gene of *Cryptosporidium* species.[13,14]

The volume of the PCR reaction mixtures was, in both cases, 50 μL, from which the DNA sample was 5 μL. In these reactions, we used primers with a concentration of 0.2 μM and 5 U Taq DNA polymerases (FIREPol).

The PCRs were run in a thermo cycler (XP Thermal Cycler Blocks) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 61/57 °C for 1 min, and 72 °C for 2 min. A final elongation step at 72 °C for 7 min was included for the complete extension of the amplified products.

#### *Electrophoresis and sequencing*

A secondary PCR product was evaluated by electrophoresis and visualized under UV light with 312 nm wavelength. Samples that were positive after sequencing were consequently compared to sequences stored in the GenBank in accordance with the genetic marker of 18 SSU rRNA gene.[15]

PCR products were directly sequenced in both directions. The sequences were aligned and completed using Chromas Pro Programme and compared to known sequences in the National Centre for Biotechnology Information GenBank database.

#### *Phylogenetic analysis*

The sequenced data were processed to form a sequence alignment for identifying similarities using MEGA6 software in subdirectory Align with CLUSTAL W option. Subsequently, the phylogenetic tree was constructed also with MEGA6 software using a Phylogeny menu and Maximum Likelihood method.

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