SZENT ISTVÁN UNIVERSITY

PROTOZOA DETECTION, IDENTIFICATION AND THE ROLE OF HUMAN ENVIRONMENT

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INTRODUCTION AND OBJECTIVES

Most of the protozoa live free in the environment and only a small part of them get in contact with plants, animals or humans. Several studies were presented, where it was described that a few protozoa cyst is enough for the sickness to emerge in the humans, however, it depends strongly on the subjects age and immune conditions. Parasitology was the last speciality where the molecular biological methods were introduced. All protozoa can be recognized by microscopy. But because of the high sensitivity and at the same time the differentiation possibility of the morphologically very similar species (or strains) today the molecular biologic methods, especially the PCR, became a very important part in the parasitological diagnostics. The molecular biologic methods have grate importance not only in parasitological diagnostics but also in epidemiology, population-genetics, in the new agent and vaccine development and above all in phylogenetic examinations. According to this, our goals were the followings:

- *Entamoeba histolytica* detection from the test materials with classic and molecular (PCR) methods with the help of the specific part of the 16S-rRNS gene coded in the parasites genome.
- Detection of *Acanthamoeba* spp. from rhizosphere of maize and lucerne by classical and modern methods.
- The sequencing and species determination from the rhizosphere isolated *Acanthmoeba* spp.
- *Giardia intestinalis* detection from the test materials with classic nested (PCR) method with the help of the specific part of the 18S-rRNS gene coded in the parasites genome.

- **MATERIALS AND METHODS**

During the job according to our goals I made a profession-science job with three known protozoa. In my study, I would like to present the elaboration of the new methods and settings.

- Detection of *Entamoeba histolytica* with real-time FRET PCR, the primers were published by Roy et al (2005). The probes I have designed with the Roche LC Probe Design Program (Gene Bank No.: X64142) with particular attention devoted to the assumed clinical diagnosis imported *E. histolytica* infection.
- In the corn and lucerne rhizosphere, I examined the *Acanthamoeba* spp. with the new real-time FRET PCR. I designed the primers and probes with the Roche LC Probe Design Program (Gene Bank No.:AF325888). The positive result was inserted in BLAST and further I made phylogenetic tree (phylogenetic analysis) with alignment programme (Multalin) according to sequence-alignment.
- *Giardia intestinalis* was examined in faecal samples from dogs with various age and gender from the kennel and breeders' origin. Then the study materials were examined and compared with classic nested PCR method.
- The experiment was made under controlled circumstances.
- The data was examined with one and two factorial variance-analysis and the correlation-interrelations were done with linear regression.

- **RESULTS**

I. *Entamoeba histolytica* identification

Between 2003 and 2008, the Department of Parasitology, of the National Center for Epidemiology received (75 stool and 2 liver abscess pus samples) with suspected amoebiasis. Microscopically, 3 out of the 77 faecal samples were declared *E. histolytica/E. dispar* positive by the Merthiolate-Iodine-Formaldehyde (MIF) concentration technique and subsequent staining with Lugol's iodine solution. By the microscopic examination, the sensitivity was lower (3.89%), because by microscopic examination a morphological isolation cannot be made between *E. histolytica* and *E. dispar*. In the Chapter2.6.2., I present that according to the applied laboratory methods (WHO, 1997) it would be very important to differentiate the two species both for therapeutic and clinical and epidemiological reasons. Therefore, alternative, more specific and more sensitive diagnostic techniques have been developed. These techniques include enzyme immunoassays (ELISA) for the rapid detection of the adhezin of *E. histolytica* in human faecal specimens. Thus, 75 stool samples were examined for *E. histolytica* by ELISA Ag Wampole^{TM}. The ELISA examination showed 4.0% sensitivity, and 3 samples were positive. The microscopic and ELISA examination had a low sensitivity, therefore the molecular biological method was applied. The molecular biological method used by us is specific because we applied two confirmed primers (Roy et al., 2005) amplified and evaluated by electrophoresis and FRET PCR method. The electrophoresis and FRET PCR showed 31.16% sensitivity and 24 positive samples (Table 1.).

Table 1. Significant differences in the results obtained with microscopy, *E. histolytica* Ag-specific ELISA test (Wampole^{TM}, Catalog No. 30404, USA), electrophoresis and FRET PCR methods.

Examination type	Positive n (%)	Negative n (%)	Total n of samples
Microscopy	3 (3.89)	74 (96.10)	77
Wampole^{TM} ELISA	3 (4.0)	72 (96.0)	75
PCR	24 (31.16)	53 (68.83)	77
FRET PCR	24 (31.16)	53 (68.83)	77

Table 1 shows that both the results and the sensitivity of the two PCR methods are equal. Therefore, I developed a highly specific and very sensitive, fast method which can evaluate 32 samples in 2 hours and which is able to detect *E. histolytica* from any secretion, abscess and punctate. The LightCycler PCR designed for the *E. histolytica* gene detects the parasite species specifically. The examination in laboratories is able to detect > 2,5 parasites /ml *E. histolytica* (Fig. 1).

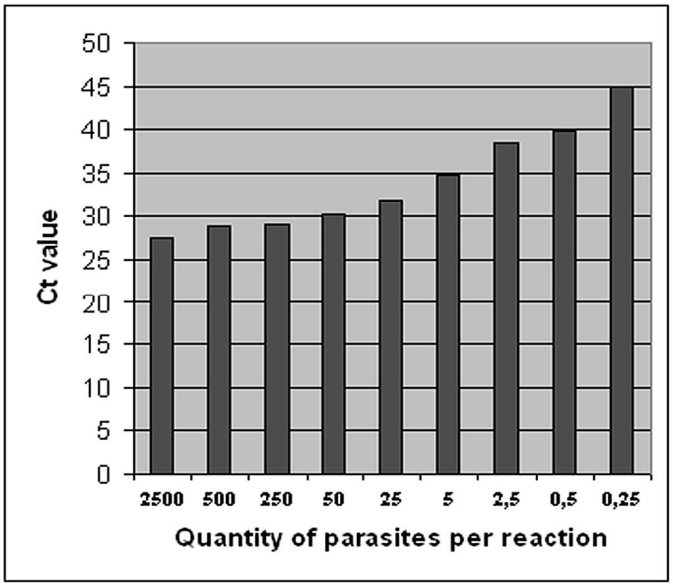


Figure. 1 Sensitivity assay variabilities experiment and positive signals (Ct values). The minimum detection limit of the *E. histolytica* FRET assay was evaluated by use of a 10-fold dilution series of DNA (2500 to 0.25 parasites in a 20µl PCR reaction volume) extracted from stool and liver abscess pus samples from four patients infected with *E. histolytica*

II. *Acanthamoeba spp.* identification

With cultivation process from the corn and lucerne rhizosphere, the free living amoebaes were detected besides the bacteria and fungi on Page agar. The amoebas from medium culture were morphologically detected as *Naegleria* and *Acanthamoeba* species. (Fig. 2).

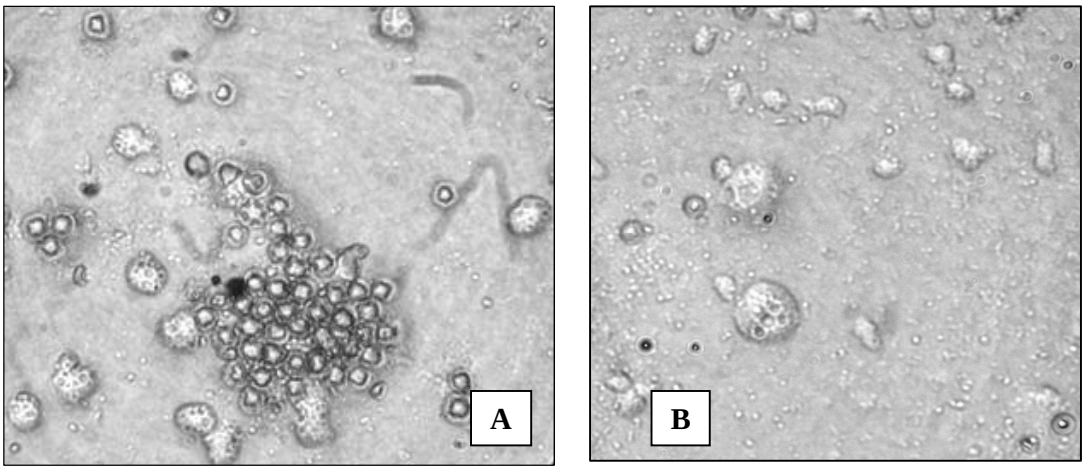


Figure 2. Photomicrograph of amoebae cysts and trophozoites with 320x (A) and 400x (B) magnification

In 1g sample taken from the corn and lucerne with *Acanthamoeba* spp. real-time FRET PCR method by melting curve with absolute quantification, I managed to determine the DNA concentration. So the KUK1-264,0db/20µl, KUK2-1,0 db/20µl, KUK3-68,0 db/20µl, KUK4-1,3 db/20µl, KUK5-2,4 db/20µl, KUK6-4,1 db/20µl, LUC1-129,0 db/20µl, LUC2-74,0 db/20µl, LUC3-46,0 db/20µl, LUC4-1,6 db/20µl, LUC5-1,6 db/20µl, LUC6-4,0 db/20µl and irrigation system (irrigation system -1liter water concentrated PVDF) 1,0 piece/20µl the *Acanthamoeba* genus was 13 positive samples. The Ut1, Ut2, Ut3, Gyep1, Gyep2, Gyep3 and the pool (1liter pool concentrated PVDF) the *Acanthamoeba* genus real-time FRET PCR provided negative results.

Acanthamoeba sp. sensitivity

The minimum detection limit of the Acanthamoeba FRET assay was evaluated by use of a 10-fold dilution series of DNA (17000 to 0.017 parasites in a 20 µl PCR reaction volume). The detection limit of *Acanthamoeba* spp. 0.17 per PCR volume was achieved. The Ct mean

values, ranged from 19.74 for 17000 parasites per PCR volume to 36.26 for 0.17 parasite per PCR volume. (Figure 3.).

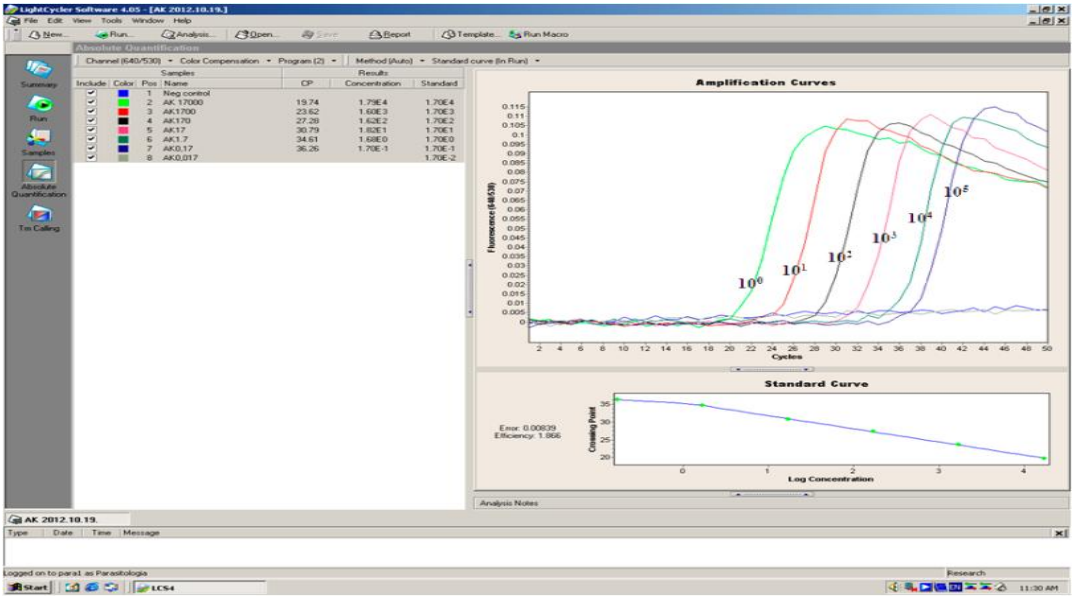


Figure 3. Sensitivity assay variabilities experiment and positive signals (Ct values). The minimum detection limit of the *Acanthamoeba* FRET assay was evaluated by use of a 10-fold dilution series of DNA (17000 to 0.017 parasites in a 20µl PCR reaction volume).

The LightCycler PCR designed for the *Acanthamoeba* spp. gene species-specificity detects the parasite. The examination in our laboratory is able > 0,017 db/20µl *Acanthamoeba* spp. detection. The correlation is significant on level p=0,05.

Acanthamoeba spp. specificity

The specificity of the method is provided by the appropriate selection of primers and probes. I compared the primers and probes with comparing sequence analysis with the known sequence data to avoid accidental homologies.

I made the real-time FRET PCR examination with *Acanthamoeba* spp. positive sample and thropozoits obtained from *Acanthamoeba* spp cultivation. All the samples and thropozoits obtained from the cultivation gave positive results, and the melting point was identical 55°C (Fig. 4.).

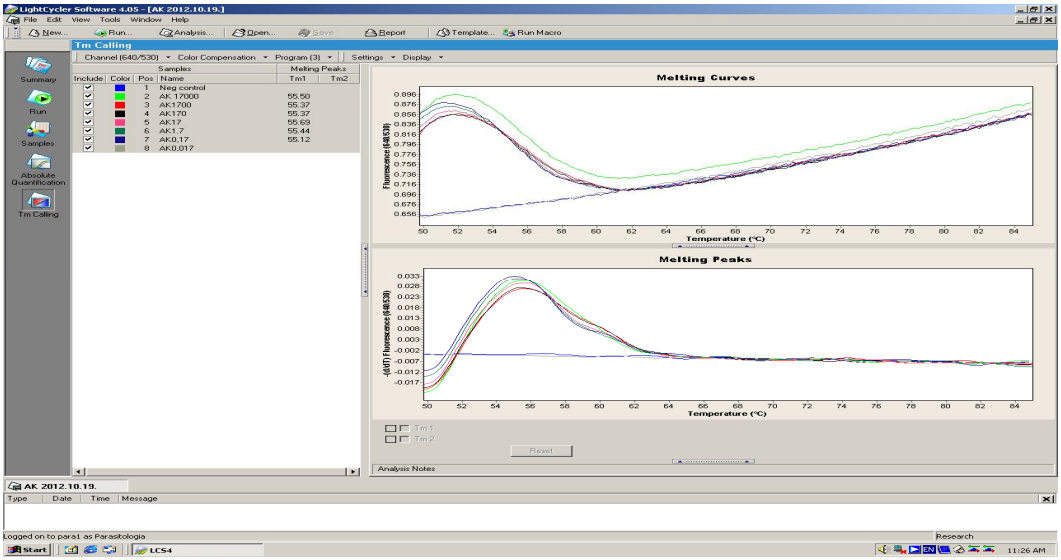


Figure 4. Melt curve analysis in the *Acanthamoeba* spp assay using the controls, showing specific Tm peaks.

The results of *Acanthamoeba* spp. sequence analysis and phylogenetic analysis

The 13 *Acanthamoeba* – positive samples, which were detected by PCR method, were sequenced to identify the species (NCBI BankIt1593841: KUK1 – KC434439; KUK2 – KC434440; KUK3 – KC434441; KUK4 – KC434442; KUK5 – KC434443; KUK6 – KC434444; LUC1 – KC434445; LUC2 –KC434446; LUC3 – KC434447; LUC4 – KC434448; LUC5 – KC434449; LUC6 – KC434450; Watering – KC434451). DNA sequences of *Acanthamoeba* reference strains from NCBI showed 95–98% similarity to all 13 PCR products. Neighbour-Joining analysis inferred relationships between the PCR products isolated from photosphere of plant and reference strains obtained from NCBI GenBank, shown in Figures 5 and 6, respectively. The most frequently identified *Acanthamoeba* genotype was T4 (n = 6) followed by T11 (n = 3), T2/T6 (n = 2), T17 (n = 1), T8, 9, 10 and T12 (n = 1). The 5 sample isolates, Kuk1, Kuk3, Kuk4, Kuk5, Kuk6 and Luc1, detected in this study were similar to genotype T4 (NCBI U07416.1), which is a widely genotype known as *Acanthamoeba castellanii*. The next 3 sample isolates, Kuk2, Luc4 and Luc5 had genotype T11 (NCBI HM036186), which is also a widely genotype *Acanthamoeba castellanii*. The other sample isolates Luc2 and Luc3 had genotype T2/T6 (NCBI AF019063.1); the Watering PVDF detected in this study had genotype T17 (NCBI JF437606.1) and the Luc6 had similarities to genotypes T8 (NCBI *Acanthamoeba tubiashi* AF019065.1), T9 (NCBI

Acanthamoeba comandoni AF019066.1), T10 (NCBI *Acanthamoeba comandoni* AF019066.1) and T12 (NCBI *Acanthamoeba healyi* AF019070.1).

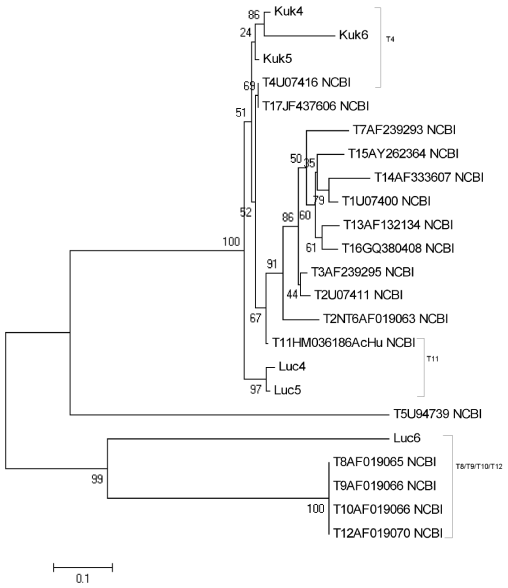


Figure 5. Phylogenetic relations of *Acanthamoeba* species PCR product Kuk1, Kuk2, Kuk3, Luc1, Luc2, Luc3, Watering PVDF and reference strains from NCBI GenBank inferred by Neighbour-Joining analysis from pair-wise comparisons (180-bp fragments)

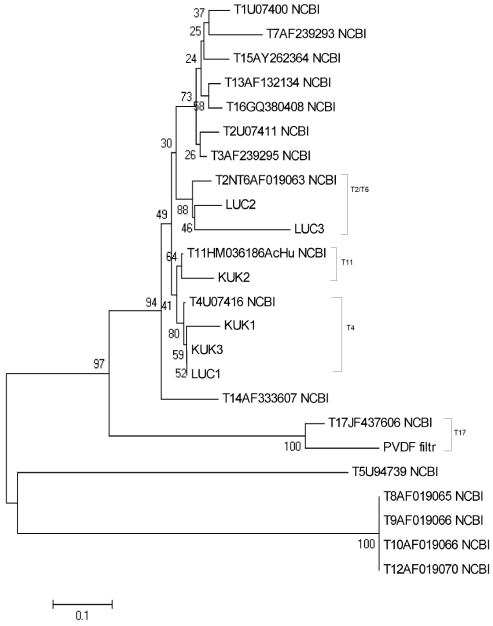


Figure 6. Phylogenetic relations of *Acanthamoeba* species PCR product Kuk4, Kuk5, Kuk6, Luc4, Luc5, Luc6 and reference strains from NCBI GenBank inferred by Neighbour-Joining analysis from pair-wise comparisons (180-bp fragments)

The *Acanthamoeba* T4 genotype can cause keratitis and encephalitis in humans, the *Acanthamoeba* T11 genotype can cause keratitis in humans; about the *Acanthamoeba* T8 and T9 genotype there is no published data as causing human infections so far, but *Acanthamoeba* T10 genotype can cause keratitis and encephalitis in humans, and finally the *Acanthamoeba* T12 genotype can cause encephalitis.

III. *Giardia intestinalis* identification

To achieve high-sensitivity detection of the *G. intestinalis* 18S-rRNA gene, I adopted the nested PCR method described by McGlade et al. (2003).

In 2006-2007, the Department of Parasitology of National Center for Epidemiology received 187 fresh faecal samples from kennel dogs. By microscopy, 14 out of the 187 (7.5%) dog faecal samples were found *G. intestinalis*-positive. Whilst a *G. intestinalis* Ag-specific coproantigen ELISA test (ProSpect Remel, USA) found 110 (58.8%) positive ones in the same set of samples. The nested PCR gave positive result in 11 cases for the 187 examined samples which sensitivity was very low (5.8%) shown in Table 2. The Figure 7. shows the distribution of the nested PCR positive samples by age-groups.

Table 2. Significant differences in 2006-2007, the results obtained with microscopy for *G. intestinalis*, and by Ag-specific coproantigen ELISA test (ProSpect Remel, USA) and nested PCR methods.

Examination type	Total n of samples	Positive n	n %
Microscopy	187	11	7,5
Giardia intestinalis coproantigen ELISA	187	100	58,8
Nested PCR	187	11	5,8

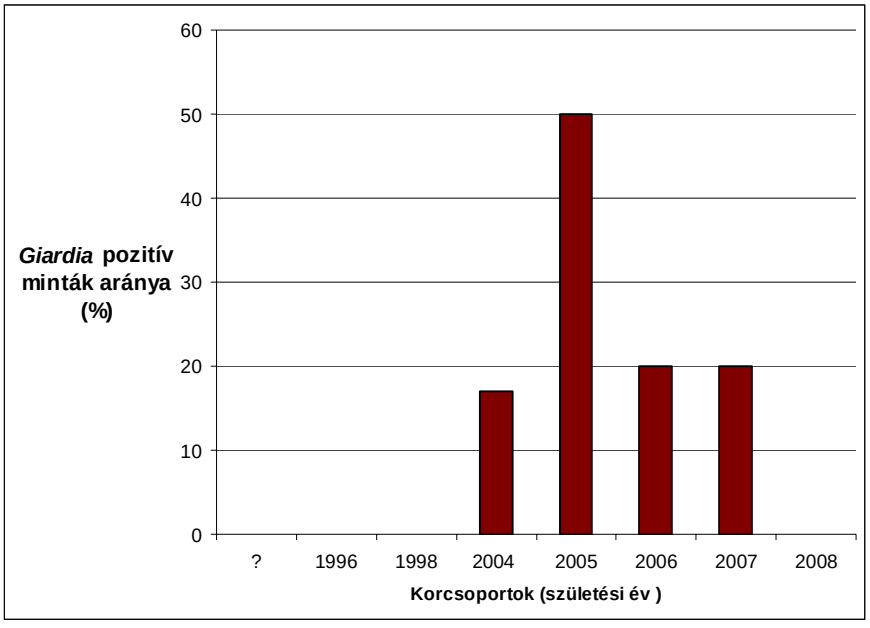
Figure 7. The number of infected dogs by age-groups. The infection rate significantly declines with the increasing age of sampled animals.

In 2008, the Department of Parasitology of National Center for Epidemiology received 40 fresh faecal samples from kennel dogs. By microscopy, 1 out of the 40 (2.5%) dog faecal samples was found *G. intestinalis*-positive. Whilst a G. intestinalis Ag-specific coproantigen ELISA test (ProSpect Remel, USA) found 21 (52.8%) positive ones in the same set of samples. The nested PCR gave positive result in 4 cases for the 40 examined samples which sensitivity was very low (17.5%) shown in Table 4. The Figure 8. shows the distribution of the nested PCR positive samples by age.

Table 4. Significant differences in 2008, the results obtained with microscopy for *G. intestinalis*, by Ag-specific coproantigen ELISA test (ProSpect Remel, USA) and nested PCR methods.

Examination type	Total n of samples	Positive n	n %
Microscopy	40	1	2,5
Giardia intestinalis coproantigen ELISA	40	21	52,8
nested PCR	40	7	17,5

Figure 8. The number of infected dogs by age groups. The infection rate significantly declines with the increasing age of sampled animals.



The results of *Giardia intestinalis* sequence analysis and phylogenetic analysis

DNA sequences of *G. intestinalis* reference strains from NCBI showed 95–98% similarity to all 18 PCR products. Neighbour-joining analysis inferred relationships between the PCR products isolated from dog stool and reference strains obtained from NCBI GenBank, shown in Figures 9 and 10, respectively.

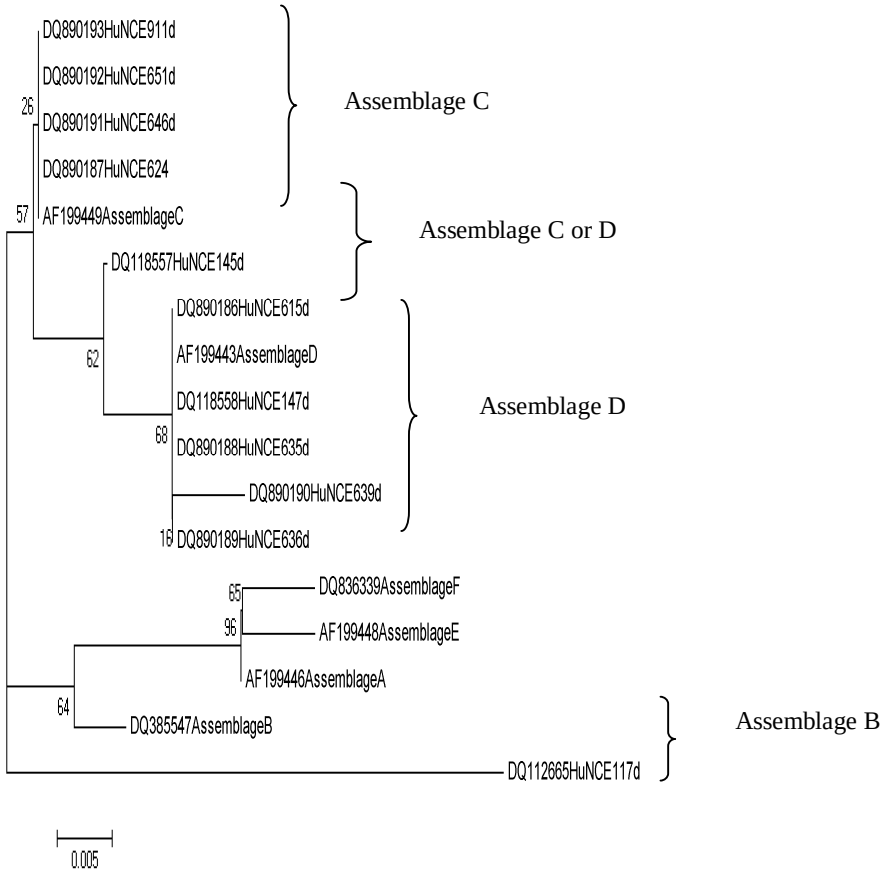


Figure 9. Phylogenetic relations of *Giardia intestinalis* species PCR product DQ112665.2, DQ118557.2, DQ118558.2, DQ890186.1, DQ890187.1, DQ890188.1, DQ890189.1, DQ890190.1, DQ890191.1, DQ890192.1, DQ8901931 and reference strains from NCBI GenBank (AssemblageA_AF199446.1; AssemblageB_DQ385547.1; AssemblageC_AF199449.1; AssemblageD_AF199443.1; AssemblageE_AF199448.1; AssemblageF_DQ836339.1) inferred by neighbour-joining analysis from pair-wise comparisons (180-bp fragments)

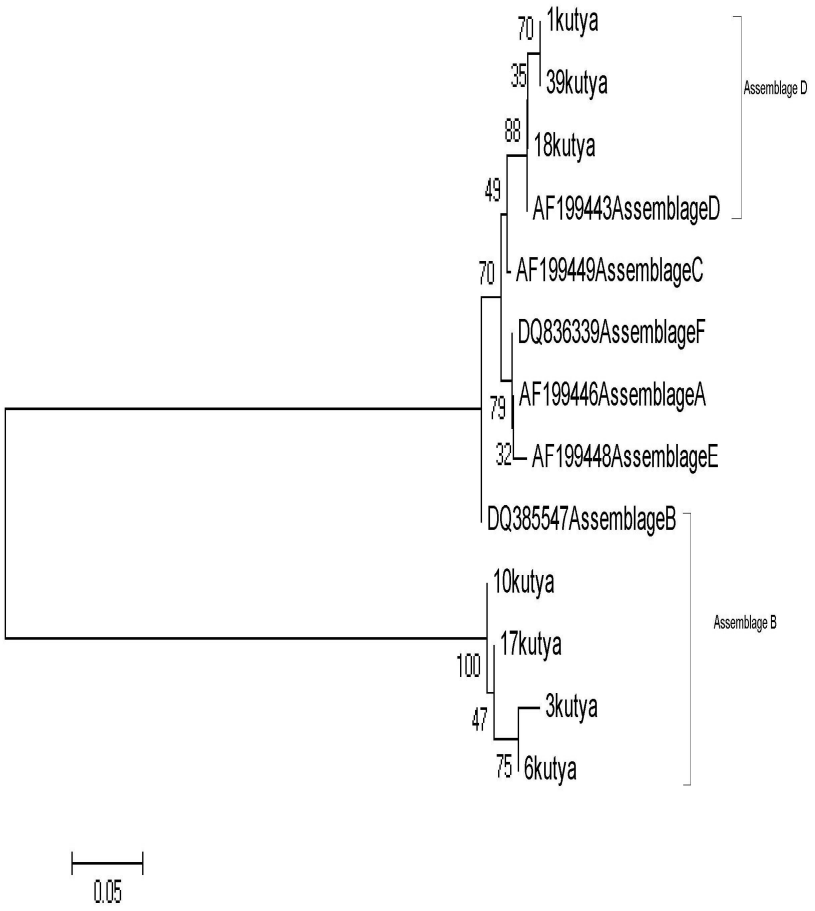


Figure 10. Phylogenetic relations of *Giardia intestinalis* species PCR product 1kutya, 3kutya, 6kutya, 10kutya, 17kutya, 18kutya, 39kutya and reference strains from NCBI GenBank (AssemblageA_AF199446.1; AssemblageB_DQ385547.1; AssemblageC_AF199449.1; AssemblageD_AF199443.1; AssemblageE_AF199448.1; AssemblageF_DQ836339.1) inferred by neighbour-joining analysis from pair-wise comparisons (180-bp fragments)

- **DISCUSSION AND CONCLUSIONS**

In accordance with the objective of my thesis, I presented the protozoa detection, identification and function in the human environment. It should be stated that a reliable diagnosis is very important for the patient to avoid the even fatal complications. It requires the early detection of infection, rapid and accurate laboratory diagnosis, and the prompt beginning of the treatment. For the early detection and treatment, the responsibility falls on the clinician, the responsibility for the laboratory workers - our duty - is to ensure the accurate and rapid laboratory diagnosis.

Expectations from the laboratory testing methods:

- High sensitivity
- The identification in the level of human pathogenic species
- Potential quantification
- Ease in use
- Speed and efficiency as possible within 1-2 hours

Most of the protozoa live free in the environment and only a small part of them get in contact with plants, animals or humans. Parasitology was the last speciality where the molecular biological methods were introduced but it has become a very important part of the parasitological diagnosis. Molecular biological diagnostic methods are also very important in epidemiology. The examined three protozoa samples with the prevalence of Hungary included domestic and imported cases.

This is the first to introduce the specific real-time PCR methods used to identify *E. histolytica* with potential for use in routine clinical practice. Obviously, *E. histolytica* has not been studied up to the end of its pathogenesis. Approximately, 500 million people have invasive amoebiasis annually, causing 100 000 deaths per year, and being the second most common cause of parasitic death in humans, with the highest incidence rates in Central and South America, Africa, and India.

The second and third objectives were successfully achieved with the detection of *Acanthamoeba* spp. from the rhizosphere of maize and lucerne by classical and modern methods. The published data in international literature, suggest that *Acanthamoeba* spp. has been isolated from rice rhizosphere. *Acanthamoeba* species in the environment have a positive impact to keep the balance with bacteria on the surface of the earth and in the rhizosphere. The *Acanthamoeba* species feed on bacteria thus regulating the population of bacteria. The aim of the study was to introduce the genotypic characterization of *Acanthamoeba* spp.

isolated from rhizosphere of maize and lucerne plants in Hungary. The FRET real-time PCR method can be safely used for detection of *Acanthamoeba* from water, soil and human samples. Therefore, the presence of *Acanthamoeba* should be considered as potential health threat associated with human activity.

In recent years, the research and the complete genome sequencing of genes have far advanced in the case of many protozoa opening new opportunities in the *Giardia* biology, diagnosis, and their monitoring. Molecular epidemiology of biochemical and molecular biology techniques were used to determine the genetic variability of the pathogen, and to clarify the control of infectious diseases in epidemiological context.

The fourth objective was the examination of G. intestinal. The aim of my study was to detect *Giardia intestinalis* from the examined samples with classic and nested PCR methods with the help of the specific part of in the protozoa genome coded 18S-rRNS gene, which could have been clarified epidemiological correlations. Developed a highly specific and very sensitive rapid method which enables the direct detection of the parasite from any secretion, abscess, liver abscess pus, the *E. histolytica, Acanthamoeba* and *G. intestinali* detection, so that the therapy of infected persons can be started very fast.

Several times I emphasized in my thesis that these infections can be significantly reduced with hand washing and compliance with the rules of hygiene.

- **NEW SCIENTIFIC RESULTS:**

- From the 77 examined samples 24 were positive for the 16S rRNA *Entamoeba histolytica* with real-time FRET PCR method on the LighCycler device and according to sequence proved to be *Entamoeba histolytica*.

- For the 16S rRNA *Entamoeba histolytica*, two different detections can be made with real-time FRET PCR method. The first one is quantitative when this result is positive or negative. The second one is Absolute Quantification because the software system is able for the automatic calculation.

- For the 18S rRNA *Acanthamoeba* sp*p.* detection I invented a real-time FRET PCR method on LighCycler device. The 13 positive samples of *Acanthamoeba* genus were registered to NCBI BankIt1593841.

- I was the first to be able to detect from ecologically balanced biotopes the presence of *Acanthamoeba* genera from the rhisosphere of maize and lucerne.

- With real-time FRET PCR method I determined the exact volume of the *Acanthamoeba* spp too.

- For the detection of *Giardia intestinalis* from dog faecal samples the nested PCR methods were also adapted compared to the microscopy Ag ELISA methods.

- I managed to detect *Giardia intestinalis* from the 227 dog faecal samples with nested PCR method. Furthermore, we studied the epidemiological correlations.

LIST OF PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

Scinetific articles (IF)

1. OROSZ E., FARKAS Á., KÖDÖBÖCZ L., BECSÁGH P., DANKA J., KUCSERA I., FÜLEKY G. (2013): Isolation of *Acanthamoeba* from the rhizosphere of maize and lucerne plants. *Acta Microbiologica et Immunologica Hungarica,* 60 (1) 29-39.p. [PUBMED: 23529297] (If**:** 0,787)

2. OROSZ E., PERKÁTAI K., KAPUSINSZKY B., FARKAS Á., KUCSERA I. (2012): Real-time PCR assay for rapid Qualitative and Quantitative detection of *Entamoeba histolytica*. *Acta Microbiologica et Immunologica Hungarica,* 59 (4) 451–460. p. [PUBMED: 23195553] (If**:** 0,787)

3. FÜZI M., PÁSZTI J., GYURIS Á., OROSZ E., MINÁROVITS J., SZÉNÁSI Z. (2008)**:** Demonstration of a protein with enhanced resistance to proteinase K in transmissible cytopathic condition elicited by cell-free lysate of free-living ameba *Naegleria gruberi*. *Structural Chemistry*, 19 (2) 203-208. p. **(**If**:** 1.433)

4. SZÉNÁSI Z., MARTON S., KUCSERA I., TÁNCZOS B., HORVÁTH K., OROSZ E., LUKÁCS Z., SZEIDEMANN Z. (2007): Preliminary investigation of the prevalence and genotype distribution of *Giardia intestinalis* in dogs in Hungary. *Parasitology Research,* 101 (1) 145-152. p. (If**:** 1.511)

Short Publications:

5. KUCSERA I., DANKA J., GLATZ K., OROSZ E. (2011): Laboratory diagnosis of human toxoplasmosis et the Department of Parasitology National Center for Epidemiology, Budapest, Hungary. *Acta Microbiologica et Immunologica Hungarica***,** 58 (Supplement) 62-63. p.

6. GLATZ K., DANKA J., OROSZ E., KUCSERA I. (2011): Foodborne human parasitoses in Hungary: diagnostic techniques at the Department of Parasitology, results from the last decade. *Acta Microbiologica et Immunologica Hungarica***,** 58 (Supplement) 31-32. p.

7. GLATZ K., DANKA J., OROSZ E., KUCSERA I. (2011): Leishmaniasis: topicalities and a review of cases detected in Hungary. *Acta Microbiologica et Immunologica Hungarica***,** 58 (Supplement) 145-146. p.

8. HORVÁTH P., KANIZSAI S., KAPÁS M., PÁSZTI J., OROSZ E., NAGY K., FÜZI M. (2009): Demonstration of a protein with enhanced resistance to proteinase-K in transmissible cytopathic conditionelicited by cell-free lysate of free-living amoeba Naegleria gruberi**.** *Acta Microbiologica et Immunologica Hungarica***,** 56 (Supplement) 170.p.

International Conferences:

9. OROSZ E., FARKAS Á., KÖDÖBÖCZ L., BECSÁGH P., DANKA J., KUCSERA I., FÜLEKY G. (2013): ISOLATION of *Acanthamoeba* from the rhizosphere of maize and lucerne plants. European Geosciences Union General Assembly 2013 (April 07 – 12.). (Absztrakt kötet).

10. KUCSERA I., DANKA J., SZÉNÁSI Z., OROSZ E., AUER H., GENCHI C. (2012): Human *Dirofilaria repens* infection in Hungary *Third European Dirofilaria Days, EDIS – European Dirofilaria Society, Olaszország, Parma* (június 21-22.) 44.p.

11. DANKA J., KUCSERA I., OROSZ E., SZÉNÁSI Z. (2012): Short history and review of relevant data of serodiagnosis of human toxocarosis in Hungary. Toxocara2012 ESCCAP – European Scientific Counsel Companion Animal Parasites *Budapest* (október 3-5) (Absztrakt kötet).

12. SZÉNÁSI Zs., FUENTES I., RUBIO J. M., MONTOYA M.A., HORVÁTH K., BECSÁGH P., OROSZ E., MARTON Sz., MÁRTON P., KUCSERA I., SZEIDEMÁNN Z. (2006): Prevalence and molecular biologic characterization of zoonotic protozoa (*Toxoplama gondii, Giardia duodenalis*) in humans and animals. International Conference of Industrial Hygiene and Occupational Medicine, The New Era of Occupational Health, Taipei, Taiwan (April 28-29.) 42-43. p.

National conferences:

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