PhD THESIS SUMMARY

Diagnostic techniques of *Encephalitozoon cuniculi* in the domestic rabbit (*Oryctolagus cuniculus*)

PhD student **Anca-Alexandra Doboși**

Scientific coordinator **Prof. Dana Liana Pusta, PhD**

I. INTRODUCTION

Microsporidia is represented by a group of eukaryotic, unicellular, obligate intracellular, spore-forming microorganisms, having vertebrates and invertebrates as hosts (Han *et al.*, 2020). *Encephalitozoon cuniculi*, the main subject of this study, is part of this category.

E. cuniculi is an eukaryotic microorganism, having features of both a protozoan and a fungus, firstly discovered in 1922 in the domestic rabbit (Wright and Craighead, 1922), but with transmissibility to numerous other species, including humans, which is why the infection may have a zoonotic potential (Magalhães *et al.*, 2022). In rabbits, transmission takes place over the horizontal route, via oral ingestion or rarely inhalatory, and the vertical route, transplacental from doe to kit (Meredith and Richardson, 2015). Two types of immunity are responsible for the organisms' protection after exposure to the pathogen, cell-mediated immunity through lymphocytes and cytokines and the humoral immunity by IgM and IgG antibodies production respectively (Latney *et al.*, 2014). Clinical presentation of infected rabbits is described mainly by neurological signs (ataxia, head tilt, nystagmus, convulsive seizures), but also renal (polydipsia, polyuria) and ocular (phacoclastic uveitis) signs to a lesser extent, however most infections have an asymptomatic presentation (Harcourt-Brown and Holloway, 2003). The treatment aims to reduce the spore load and inflammation caused by the microorganism, with the help of fenbendazole, anti-inflammatories and supportive therapy, but no form of medication assures a full recovery (Latney *et al.*, 2014). For this reason, prophylactic measures are of great importance for reducing infection spread, such as periodical testing of large rabbit populations, preventive administration of fenbendazole and thorough disinfection of the environment (Meredith and Richardson, 2015).

The diagnosis of *E. cuniculi* in rabbits cannot be based solely on clinical presentation, which is unspecific, therefore serological, histopathological and molecular genetics techniques are currently the main methods of identifying the microorganism or its antibodies (Latney *et al.*, 2014). Considering its latent and zoonotic character, pathogen detection in large populations is important for establishing the diagnosis, in order to raise awareness among clinicians and rabbit owners, as well as for taking the necessary preventive measures.

The work hypothesis is represented by the importance of *E. cuniculi* diagnosis, using multiple methods, which is a widely spread microsporidian among rabbit populations, very often in a latent state, that can also present zoonotic risk.

The objectives of the research were represented by *E. cuniculi* diagnosis using clinical diagnostic tools, serological methods and molecular genetics techniques, as well as analytical comparison of the obtained results and presentation of some case studies.

Firstly, clinical examination and performance of certain paraclinical investigations (blood tests, coproparasitological exam, imaging investigations), especially in rabbits

presenting clinical signs specific to encephalitozoonosis, had the aim of evaluating their health status and the importance of paraclinical tests in a clinical context.

Serological diagnosis represented one of the main objectives, this being performed by a qualitative ELISA with IgG anti-*E. cuniculi* antibody detection from rabbit blood sera and the analysis of the obtained results.

Diagnosis using molecular genetics techniques was the second main objective. Nested PCR and qPCR techniques for specific *E. cuniculi* DNA identification were performed, using samples of urine, feces and organs, followed by results analysis.

Finally, comparative studies between the serological and molecular genetics techniques were performed, in order to evaluate their efficacy and to identify the best method of microsporidian diagnosis.

A secondary objective of this study consisted of case studies presentation for highlighting the importance of *E. cuniculi* diagnosis in rabbits, no matter their clinical status.

II. PERSONAL CONTRIBUTION

The chapter Personal contribution from the thesis entitled "**Diagnostic techniques of *Encephalitozoon cuniculi* in the domestic rabbit (*Oryctolagus cuniculus*)**" includes four studies with the main objective of pathogen identification by clinical and paraclinical exams, serological (ELISA) and molecular genetics (nested PCR, qPCR) methods. Moreover, the case studies had the objective of highlighting the importance of *E. cuniculi* diagnosis for rabbits manifesting clinical signs, mainly neurological signs, as well as for asymptomatic ones. Domestic rabbits owners were informed about the study's objectives, signed a written consent for participation approval and results were communicated to them.

1. Study 1 - Clinical diagnosis and paraclinical tests

The main aim of this study was performing a clinical evaluation and certain paraclinical tests of the rabbits, especially for the ones manifesting specific signs of encephalitozoonosis. The secondary objective was to evaluate the rabbits' clinical status in accordance with the paraclinical tests performed, in order to identify their applicability, especially in the context of an acute infection. A final aim was represented by the implementation and the analysis of the therapeutic management where possible.

In this study a total of 370 domestic rabbits (*Oryctolagus cuniculus*) were included for a clinical evaluation. In some cases, paraclinical investigations were performed, which included blood biochemistry, coproparasitological exam, head CT and radiographic exam.

Conclusions:

1. Following clinical examination, out of the 370 rabbits, 8.65% (32/370; IC 95%: 6.19-11.95) rabbits were categorised into the symptomatic group, presenting neurological and renal signs, while 91.35% (338/370, IC 95%: 88.05-93.81) were asymptomatic, either clinically healthy or presenting other unspecific pathologies.
2. Biochemical blood analysis revealed parameters coinciding with dehydration and liver/digestive impairment (hypoproteinemia, hypoalbuminemia, hyperglobulinemia, hypernatremia, low BUN, high ALP, high GLY), as well as with renal impairment (hypercalcemia, hypophosphatemia, hyperkalemia).
3. By coproparasitological exam parasites such as *Eimeria* spp., *Passalurus* spp. and *Trichostrongylus* spp. were identified, asymptomatic rabbits presenting a high prevalence of 50.29% (88/175; IC 95%: 42.95-57.61) and without any correlation between endoparasites and the predisposition of *E. cuniculi* infection.
4. Imagistic investigations, especially CT scan, proved to be useful in evaluating the clinical state of the patients manifesting clinical neurological acute signs, as well as for excluding other differential diagnoses, such as otitis media/interna.
5. Even if these paraclinical investigations do not offer an *E. cuniculi* certain diagnosis, they show importance in regards to management conduct of the patient for the clinician.
6. The main recommendation for rabbits with a positive *E. cuniculi* diagnosis, both symptomatic and asymptomatic, is to initiate the therapeutic protocol with fenbendazole, dosed 20 mg/kg/day orally for 28 days, in order to maximize the chances of improving the clinical state.

2. Study 2 - Serological diagnosis of *Encephalitozoon cuniculi*

The main aim of this study was the diagnosis using a qualitative ELISA to detect specific anti-*E. cuniculi* IgG sera antibodies from rabbits in the North-Western region of Romania and the statistical analysis of the results in relation to specific parameters of the different rabbit groups included in the study (age, sex, season of sampling, rearing system, clinical status, vaccination status and county of origin).

In this study a total of 351 domestic rabbits were included. The materials included supplies for blood collection and serum expression, materials for the serological ELISA processing (kit from Medicago, Uppsala, Sweden) and equipment for reading results (absorbance reader Bio-Rad PR2100 Microplate Reader, Bio-Rad Laboratories, USA).

Conclusions:

1. The total prevalence obtained by the serological ELISA technique for rabbits in North-Western Romania was 43.02% (151/351, IC 95%: 37.94-48.25).
2. Significant differences were statistically obtained in relation to age, season of sampling, BCS, clinical status and county of origin.
3. ELISA was the serological method chosen due to its feasibility for processing a large load of samples.

4. For the adult rabbit group a significantly higher prevalence was recorded 48.23% (136/282, IC 95%: 42.46-54.04) compared to the young group 21.74% (15/69, IC 95%: 13.64-32.82).
5. Samples collected in the spring and autumn seasons revealed the highest positivity, 65.57% (40/61, IC 95%: 53.05-76.25) and 45.27% (67/148, IC 95%: 37.47-53.31) respectively.
6. Individuals with the presence of clinical signs specific to encephalitozoonosis proved a relevantly higher seroprevalence (61.29%, IC 95%: 43.82-76.27) compared to asymptomatic rabbits (41.25%, IC 95%: 35.99-46.72).
7. For the rabbits with the BCS of 2/5 (55.56%; 5/9, IC 95%: 26.67-81.12) and 4/5 (45.24%; 19/42, IC 95%: 31.22-60.05) the highest seropositivity was recorded.
8. In the rabbits' counties of origin Alba, Cluj and Bistrița-Năsăud the highest and close positivity of 51.52% (34/66, IC 95%: 39.71-63.15), 47.95% (82/171, IC 95%: 40.59-55.40) and 46.94% (23/49, IC 95%: 33.70-60.62) respectively, was recorded.
9. Serological diagnosis using the IFAT method offered a seropositivity of 53.85% (7/13, IC 95%: 29.14-76.79), the majority of these results (12/13) being comparatively identical to those performed using ELISA.
10. Application of the serological ELISA method is recommended as a screening diagnosis for large rabbit populations, as well as for the analysis of establishing the therapeutic and prophylactic necessary measures.

3. Study 3 - Diagnosis of *Encephalitozoon cuniculi* using nested PCR

The main objective of this study was the diagnosis using molecular genetics nested PCR technique to identify specific *E. cuniculi* DNA from excretions and tissues, from pet and farm rabbits of the North-Western region of Romania and wild european rabbits of Satu-Mare county. The secondary aim was the comparison of three different work protocols, using the primers MSP1_for and MSP2A_rev for the first PCR and the primers MSP3_for and MSP4A_rev for the second PCR (Katzwinkel-Wladarsch *et al.*, 1996), the primers P2 and M2 for the first PCR and the primers Ence_549_forward and Ence_549_reverse for the second PCR (Csokai *et al.*, 2009), and the primers MK5 and MK6 for the first PCR and the primers K4 and K3 for the second PCR (Kimura *et al.*, 2013). The third objective was represented by comparison of the results obtained in relation to specific parameters (age, sex, season of sampling, rearing system, clinical status, vaccination status and county of origin), to observe the prevalence according to the sample type tested.

In this study a total of 242 rabbits were included and the necessary materials were divided into supplies for excretion (urine, feces) and organ (brain, eye lens, kidney, urinary bladder, liver, lungs, heart, spleen) collection, materials for DNA extractions (Maxwell® RSC Genomic DNA Kit, QIAamp Fast DNA Stool Mini Kit, E.Z.N.A. Stool DNA Kit, ADN ReliaPrep

gDNA Tissue Miniprep System, DNeasy® Blood & Tissue Kit) and the subsequent amplifications, as well as equipment used in the molecular testing (DNA automatic extractor Maxwell® RSC Instrument, Thermal Cycler Bio-Rad T100 şi UV Cleaver transilluminator).

Conclusions:

1. Nested PCR is a widely spread method for *E. cuniculi* diagnosis using different sample types, such as urine, feces and organs, the work protocol described by Katzwinkel-Wladarsch *et al.* (1996) revealing conclusive results in this study (Fig. 18).

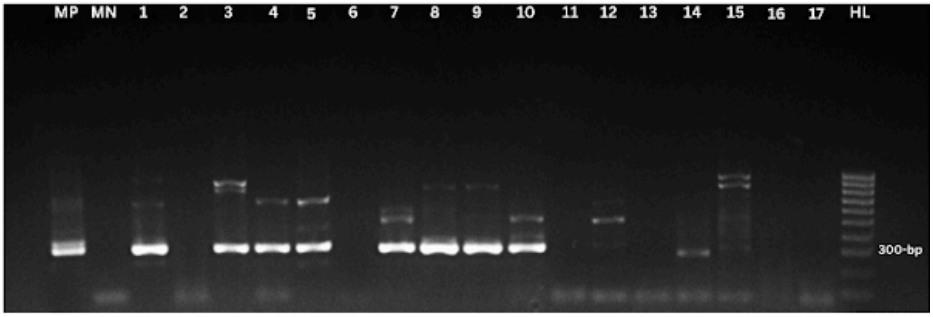

Fig. 18. Gel agarose electrophoresis 1.5%. Positivity shown for samples 1, 3, 4, 5, 7, 8, 9, 10 by the presence of a band at 300-bp level. MP - positive control, MN - negative control, HL - HyperLadder™. (Original)

2. The total prevalence of nested PCR from all sample types of 242 rabbits was 45.45% (110/242; IC 95%: 39.30-51.75).
3. *E. cuniculi* prevalence by nested PCR:
   - of urine was 27.27% (15/55, IC 95%: 17.28-40.23), with significant differences in relation to the rearing system and BCS;
   - from feces was 37.50% (72/192, IC 95%: 30.96-44.53), with relevant differences in relation to age, sex, season of sampling, rearing system, BCS, clinical status and county of origin;
   - from organs was 61.54% (32/52, IC 95%: 47.02-74.70), with significant differences in relation to sex, season of sampling, rearing system, clinical status and county of origin.
4. Nested PCR applied on the different types of organs recorded a positivity of 30.56% (11/36, IC 95%: 18.00-46.86) for the urinary bladder, this organs' testing being the first report in the specialized literature.
5. The present study identified the *E. cuniculi* presence in organs from European wild rabbits, for the first time in our country, demonstrating that wildlife is a potential reservoir of *E. cuniculi*.
6. Overall, positivity of these samples proved relevant for the following rabbit groups: juveniles, asymptomatic, housed in family farms.

4. Study 4 - Diagnosis of *Encephalitozoon cuniculi* using Real-Time PCR

The main aim of this study was the diagnosis using the molecular genetics Real-Time PCR or qPCR technique of specific *E. cuniculi* DNA detection from excretions and organs of domestic rabbits. The secondary objective was represented by the comparison of qPCR results from excretions and organs with other studies, in order to evaluate the efficacy of this method for the sample types tested.

A total of 31 domestic pet and farm rabbits were included in this study. Materials necessary consisted of supplies for excretions (urine, feces) and organ (brain, eye lens, kidney, urinary bladder, liver, lungs, heart, spleen) collection, materials required for PCR processing (DNA extraction kits: Maxwell® RSC Genomic DNA Kit, QIAamp Fast DNA Stool Mini Kit, E.Z.N.A. Stool DNA Kit, ADN ReliaPrep gDNA Tissue Miniprep System, DNeasy® Blood & Tissue Kit) and equipment for performing the molecular diagnosis (Azure Cielo™ Real-Time PCR System).

Conclusions:

1. The total prevalence using qPCR from all types of samples (n=122), represented by urine, feces and organs tested from 31 rabbits, was 48.39% (15/31, 95% CI: 31.97-65.16).
2. qPCR of urine and feces samples from 10 rabbits showed positivity for 4 and two rabbits respectively.
3. Positivity by qPCR from organs of 26 rabbits was 53.85 % (14/26, IC 95%: 35.46-71.24).
4. The urinary bladder revealed a positivity of 35.29% (6/17; IC 95%: 17.31-58.70) using qPCR, representing the first report in the specialized literature.
5. qPCR analysis of a larger sample load is necessary for applying a statistical analysis.

5. Study 5 - Comparative evaluation of the serological (ELISA) diagnostic methods and molecular genetics (nested PCR, qPCR) techniques for the identification of *Encephalitozoon cuniculi*

The main aim of this study was the performance of a comparison between the results of the serological and molecular genetics methods for *E. cuniculi* identification, correlated with the rabbits' clinical data. The secondary objective was to evaluate the methods' efficacy, as well as identifying the main risk factors involved in the exposure to the microorganism and the correlation of the diagnosis usefulness in the clinical context of the patients.

In this study a total of 381 rabbits were included, from which 378 domestic and 3 European wild rabbits. Serological diagnosis was carried out for 351 of the rabbits, while diagnosis using molecular genetics techniques was performed for 242 individuals, from samples of urine, feces or organs. All materials mentioned in the previous studies were

used to fulfill this study's objectives.

Conclusions:

1. Comparative analysis revealed a prevalence of 58.96% (125/212, IC 95%: 52.24-65.37) using the serological ELISA diagnosis and a positivity of 43.40% (92/212, IC 95%: 36.90-50.13) using the molecular nested PCR diagnosis from urine, feces or organs.
2. Significant differences between the positivity of the ELISA and nested PCR methods were found in relation to the rabbits' age and clinical status.
3. Seropositivity was higher for the adult rabbit group (69.57%, 112/212) and the symptomatic group (70.83%, 17/212), while the molecular diagnosis proved a higher positivity for the young group (60.78%, 31/212) and the asymptomatic individuals (44.68%, 84/212).
4. Compared to nested PCR as the golden standard method, a sensitivity of 58.7% and a specificity of 40.83% for the ELISA technique was identified.
5. By a risk factor assessment according to the results of ELISA (n=351) and nested PCR (n=242), rabbits' age and season of sampling proved statistical relevance in relation to seropositivity.
6. Comparison between nested PCR and qPCR methods from urine, feces or organs revealed a prevalence of 54.84% (17/31) and 48.39% (15/31) respectively, without any significant differences between the rabbit groups analysed.
7. Comparative analysis of the molecular techniques nested PCR and qPCR showed positivity in favor of the first method, but the reduced sample load indicates a need for further studies.
8. Compared to nested PCR as the golden standard method, a sensitivity and specificity of qPCR was established at 58.8% and 64.3% respectively.
9. By testing of the urine and urinary bladder using nested PCR similar results were obtained (13/20), but more studies for certifying the sensitivity of these sample types are essential.
10. Comparative analysis of all diagnostic methods performed for 6 rabbits proved qPCR from organ samples as the method with a maximum positivity, thus suggesting the biggest efficacy.

6. Study 6 - Case studies

Within this chapter a presentation of certain rabbit clinical cases was made, with or without clinical signs specific to encephalitozoonosis in relation to the results of the diagnostics performed. The 4 types of cases selected for presentation were: positive case with symptomatology present, for establishment of a certain diagnosis; positive case with the absence of clinical signs, for infection screening; negative case with symptomatology present, to establish a differential diagnosis; negative case with the absence of clinical signs, for infection screening.

III. GENERAL CONCLUSIONS AND RECOMMENDATIONS

The aim of this study focused on conducting a comprehensive study of *Encephalitozoon cuniculi* in domestic and wild rabbits from the North-Western region of Romania, more exactly by diagnosing the pathogen using serological and molecular genetics methods, as well as comparing their results and identifying these methods' efficacy, which generated the following conclusions:

1. The clinical study of the rabbits identified a prevalence of 8.40% (32/381; IC 95%: 6.01-11.62) of patients with clinical signs specific to encephalitozoonosis, mainly manifested through neurological signs (ataxia, head tilt, nystagmus, convulsive seizures with/without rolling), but also renal signs (urinary incontinence, polydipsia, polyuria, anorexia). Paraclinical investigations did not offer significant information on the direct diagnosis of *E. cuniculi*, but these remain a useful tool in the context of acute disease evaluation and for the exclusion of certain differential diagnoses.
2. *E. cuniculi* identification was achieved using serological and molecular genetics diagnostic methods.
3. Serological diagnosis performed using the ELISA method led to obtaining the following results:
   - 3.1. A total prevalence of 43.02% (151/351, IC 95%: 37.94-48.25) in domestic rabbits, with significant statistical differences in relation to age, season of sampling, BCS, clinical status and county of origin.
   - 3.2. Based on these results, the rabbit groups more prone to seropositive results were the adults, the spring and autumn seasons of sampling, the groups with a BCS out of normal range and the ones with clinical signs specific to encephalitozoonosis.
   - 3.3. The serological ELISA method proved good feasibility for testing of a large rabbit population and as a qualitative method, while comparative results of the IFAT method showed a good correlation of results.
4. Diagnosis using molecular genetics techniques aimed for the identification of specific *E. cuniculi* DNA by PCR (nested PCR, qPCR) from samples of urine, feces or organs, from both domestic and European wild rabbits, obtaining the following results:
   - 4.1. Nested PCR showed a total prevalence of 45.45% (110/242; IC 95%: 39.30-51.75). Between the different sample types tested, the highest prevalence was identified for organs 61.54% (32/52, IC 95%: 47.02-74.70), owed to the fact that parasitic spore elimination through urine and feces is intermittent, therefore these samples present a lower probability of showing positivity.
   - 4.2. The nested PCR work protocol described by Katzwinkel-Wladarsch *et al.* (1996) offered conclusive results, by using the primers MSP1_for and MSP2A_rev for the first PCR and the primers MSP3_for and MSP4A_rev for the second PCR.
   - 4.3. Using nested PCR for *E. cuniculi* identification, a statistical relevance was

found for the following groups: juveniles, asymptomatic, originating from family farms and sampled in the summer and winter seasons. These results highlight the importance of this diagnosis, which can identify specific DNA even in a latent state, without a clinical manifestation of the animal, as well as for emphasizing the infection status of rabbits destined for meat consumption and the related risks.

4.4. The qPCR technique revealed a total prevalence of 48.39% (15/31, 95% CI: 31.97-65.16), similar to the one by nested PCR, but without the possibility of running a statistical analysis due to small sample size.

4.5. Both molecular genetics techniques studied showed positivity for the urinary bladder testing, 30.56% (11/36, IC 95%: 18.00-46.86) by nested PCR and 35.29% (6/17; IC 95%: 17.31-58.70) by qPCR respectively, which is also reported for the first time. Testing from this sample type offers a new approach on the diagnosis methods of *E. cuniculi*.

5. Comparative evaluation of serological and molecular genetics methods results proved the following:

5.1. The ELISA method revealed a higher seroprevalence (58.96%, 125/212) compared to the positivity by nested PCR (43.40%, 92/212). The first technique showed a significantly higher prevalence for the adult and symptomatic rabbit groups, while the latter showed significantly higher positivity for the young and asymptomatic groups. These results do not state one of the methods to be more sensitive than the other, but they demonstrate the applicability of each depending on the availability of the sample types to be tested, as well as the dependence of the infection stage in relation to the patient's clinical evolution.

5.2. In relation to the seroprevalence, rabbits aged over 4 months old proved to be 4.5 times more prone to showing positive results than juveniles, while testing of blood sampled in the winter season presented significantly lower chances of seropositivity compared to those collected in spring. Comparatively, samples collected in the summer had a significantly higher chance of showing positivity by nested PCR, compared to those sampled in spring. Once again, these show *E. cuniculi*'s diverse nature depending on the diagnosis type performed and the sample type used.

5.3. By comparing the results of nested PCR and qPCR, a prevalence of 54.84% (17/31) and 48.39% (15/31) respectively was observed, positivity being in favor of the first technique.

5.4. Compared to nested PCR as a reference method, sensitivity and specificity were slightly higher for qPCR than for ELISA, however both methods remain useful.

6. The case studies highlighted the importance of *E. cuniculi* diagnosis in rabbits with an acute, symptomatic infection, in the context of differential diagnosis, as well as for the ones with no apparent clinical signs, as screening.

Recommendations:

1. For the antemortem diagnosis of *E. cuniculi* we recommend the serological methods of specific antibody detection, blood collection being quick and easy and the identification of pathogen exposure showing success.
2. For a good screening of large rabbit populations we recommend the serological diagnosis using the ELISA method, this being a feasible technique for a large sample load and a useful tool in controlling *E. cuniculi* spread.
3. For the postmortem *E. cuniculi* diagnosis we recommend testing of the predilection organs, more exactly the brain, eye lens, kidney and urinary bladder using nested PCR, this proving significant results and with greater precision, even in the absence of specific clinical signs antemortem.
4. Molecular testing from urine and fecal samples can lead to false negative results, due to the intermittent spore shedding, which is why *E. cuniculi* identification from these sample types is not recommended.

IV. ORIGINALITY AND INNOVATIVE CONTRIBUTIONS OF THE THESIS

1. The present thesis represents the first report in the specialized literature regarding *E. cuniculi* prevalence in the North-Western region of Romania, through a complete study (clinical and paraclinical investigations, serological methods and molecular genetics techniques) on domestic rabbits.
2. In the present study, *E. cuniculi* identification from organs originating from European wild rabbits, using molecular genetics techniques, was reported for the first time.
3. An element of originality in this thesis is represented by the performance of the serological and the molecular genetics diagnosis, as well as by the comparison of their results.
4. The present study aimed at molecular testing of the urinary bladder and its first report in the specialized literature regarding positivity by both nested PCR and qPCR.
5. The high positivity of asymptomatic rabbits, as well as for the ones reared as pets and in family farms are important results, especially in the context of *E. cuniculi*'s zoonotic character.
6. The analysis of *E. cuniculi* diagnosis results depending on the season of sampling offered important results, spring and autumn showing a significantly higher positivity for anti-*E. cuniculi* IgG antibodies by the ELISA method and summer by the nested PCR method, with the identification of specific *E. cuniculi* DNA.
7. Another innovative contribution is the practical applicability of the *E. cuniculi* diagnosis methods presented in this research, through serological and molecular

genetics techniques within the Laboratory of Molecular Genetics, Department of Genetics and Hereditary Diseases within FMV - USAMV Cluj-Napoca and the provision of cost-effective services to rabbit owners and breeders.

8. The results of the present study contribute to raising awareness of the owners in regards to *E. cuniculi* infection risks among rabbit populations in Romania and highlight the importance of implementing prevention measures, meant to limit the spread of the pathogen to both animals and humans.

Selective references

1. Csokai, J., Joachim, A., Gruber, A., Tichy, A., Pakozdy, A., & Künzel, F. (2009). Diagnostic markers for encephalitozoonosis in pet rabbits. Veterinary Parasitology, 163(1-2), 18-26.
2. Han, B., Takvorian, P. M., & Weiss, L. M. (2020). Invasion of host cells by microsporidia. Frontiers in microbiology, 11, 172.
3. Harcourt-Brown, F. M., & Holloway, H. K. R. (2003). *Encephalitozoon cuniculi* in pet rabbits. Veterinary Record, 152(14), 427-431.
4. Katzwinkel-Wladarsch, S., Lieb, M., Heise, W., Löscher, T., & Rinder, H. (1996). Direct amplification and species determination of microsporidian DNA from stool specimens. Tropical Medicine & International Health, 1(3), 373-378.
5. Kimura, M., Aoki, M., Ichikawa-Seki, M., Matsuo, K., Yagita, K., & Itagaki, T. (2013). Detection and genotype of *Encephalitozoon cuniculi* DNA from urine and feces of pet rabbits in Japan. Journal of Veterinary Medical Science, 75(8), 1017-1020.
6. Latney, L. T. V., Bradley, C. W., & Wyre, N. R. (2014). *Encephalitozoon cuniculi* in pet rabbits: diagnosis and optimal management. Veterinary medicine: research and reports, 169-180.
7. Meredith, A. L., & Richardson, J. (2015). Neurological diseases of rabbits and rodents. Journal of Exotic Pet Medicine, 24(1), 21-33.
8. Wright, J. H., & Craighead, E. M. (1922). Infectious motor paralysis in young rabbits. The Journal of Experimental Medicine, 36(1), 135.