



DETECTION AND DIAGNOSIS OF ENCEPHALITOOZONOSIS IN RABBITS UNDER UKRAINE-SPECIFIC CONDITIONS

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ABSTRACT

For the first time data on detection and spread of encephalitozoonosis in rabbits under Ukraine-specific conditions has been obtained. The disease in rabbits was observed over the year. Encephalitozoonosis in rabbits manifested itself asymptotically, acutely, and chronically. Asymptomatic course is the most common. In clinical terms encephalitozoonosis manifested itself as severe nervous disorder, defeat of the organs of sight and urinary system.

For the first time in Ukraine, investigation of rabbits afflicted by encephalitozoonosis was conducted with immune ferment method (ELISA) and it was established that the extensiveness of encephalitozoonosis infestation in rabbits reached 34 %. Groups with low ($4,97 \pm 1,47$ absorbance units), medium ($16,00 \pm 6,14$ absorbance units), and high ($162,72 \pm 58,00$ absorbance units) titer and clinically sick animals whose level of antibodies was the highest ($391,50 \pm 8,10$ absorbance units) were singled out from among the rabbits with positive reaction.

Histological investigations revealed sero-hemorrhagic leptomeningitis and focal purulent encephalitis. The detected changes in the organs were caused by reproduction of the parasites in the form of spores.

INTRODUCTION

Encephalitozoonosis is a wide-spread infestation of rabbits. The disease poses danger for productive, fancy, wild, and laboratory populations of rabbits [Keeble E., Shaw D. 2006; Dipineto L. et al. 2008; Valencakova A. et al. 2008; Okewole E. 2008; Cray C. et al., 2009; Ozkan O. et al., 2011, Berezovsky A. V., Levytska V. A. 2012].

Encephalitozoonosis (syn.: Encephalozoonosis, *Encephalitozoon (nosema) cuniculi*) is zoonotic protozoonosis predominantly with chronic or asymptomatic progress. It is caused by small obligate intracellular parasite *Encephalitozoon cuniculi* of *Encephalitozoon* genus which belongs to microsporidia and predominantly affects the central nervous system (brain and spinal cord), and – kidneys, liver, spleen, heart, lungs, and eyes of the rabbits. The other two species of this genus – *E. hellem* and *E. intestinalis* are also able to infest mammals [Harcourt-Brown F. 2003; Jordan C. 2005; Valencakova A. 2008; Kunzel F. 2009; Csokai J. 2010; Keeble E. 2011; Sokolova O. et al. 2011].

It is known that encephalitozoonosis afflicts people for whom the source of invasion are

domestic animals, polluted drinking water, or other sick people. However, the main role is played by rabbits as the source of the pathogen invasion [Koudela B. et al. 1999; Deplazes P. et al. 2000; Halanova M. et al. 2003; Snowden F. et al. 2004; Mathis A. et al. 2005; Ditrich O. et al. 2011; Malcekova B. et al. 2011].

In the populations of productive and laboratory rabbits the invasion is widely spread and it affects from 34 to 68 % of the local livestock [Ewringmann A., Göbel T. 1999; Harcourt-Brown F., Holloway H. 2003; Ebrecht D., Müller E. 2004, 20. Levytska V. A., Berezovskyy A. V. 2013]. The latest reports argue that the disease started to appear in infancy rabbits, which is confirmed by the presence of specific antibodies to *E. cuniculi* in the blood of more than 52 % of clinically healthy rabbits [Keeble E., Shaw D. 2006]. The research findings are of great significance as the number of registered instances of disease in people in Europe, North and South America, Africa, and Australia is steadily growing [Mathis A., 2005; Gruber A., 2009]. *E. cuniculi*, is a conditionally pathogenic microorganism posing a threat for people with weakened immune systems including HIV-infected individuals who take immune suppressor preparations, and during organ transplantation [Didier P. et al. 2000; Mathis A. et al. 2005; Flock U. 2010; Sieg J. 2014].

A number of researchers are convinced that *E. cuniculi* is one of the main problems of rabbit breeding. The majority of experts tend to think that the final diagnosis of encephalitozoonosis during the life time is difficult to establish since diagnosis of protozoasis, prevention and treatment are not clarified and not sufficiently developed [Meyer-Breckwoldt A. 1996; Ewringmann A., Göbel T. 1999; Suter C. et al. 2001; Harcourt-Brown F. 2003; Künzel F. et al. 2008; Künzel F., Joachim A. 2010, Levytska V. A., Berezovskyy A. V. 2013].

MATERIALS AND METHODS

The experimental part of the work was performed over 2013–2016 in the laboratory of the Department of Infectious and Parasitic Diseases of the Faculty of Veterinary Medicine at Podillya State Agrarian Engineering University (Kamianets-Podilskyi) and the State Scientific Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Examination (Kyiv). Histological investigation was conducted in the laboratory of Normal and Pathological Morphology and Forensic Veterinary Department of the Lviv National University of Veterinary Medicine and Biotechnologies named after S. Z. Gzhytskyi (Lviv).

The production test of the research findings was carried out on the farms and the adjoining households in Ukraine.

Rabbits of different breeds and ages, kept under different rearing conditions were examined. Rabbits from 6 weeks to 3 years of age were subjected to research.

In total 2563 rabbits were surveyed.

At the first stage of research the spread of encephalitozoonosis in rabbits on the farms of different ownership forms was investigated.

Routine preventive measures were observed during parasitological examination with parasitosis treatment schemes taken into consideration both in the past and current seasons.

At the second stage the effectiveness of laboratory diagnostic methods for encephalitozoonosis in rabbits was determined. For intravital diagnosis 88 samples were examined with enzyme immunoassay (ELISA) blood serums from rabbits. *Encephalitozoon cuniculi* ELISA test-system produced by Medicago AB of Sweden was used. For this test-system antibodies to the encephalitozoonosis pathogen in blood serum are detected in a dilution greater than 1:40. Blood for examination was sampled from clinically healthy rabbits and rabbits with clinical signs of encephalitozoonosis coming from different gender-age groups.

At the third stage of research the influence of encephalitozoonosis pathogen on the organism of rabbits was investigated. Pathological changes in the organs of rabbits, characterized by encephalitozoonosis were determined and microsporidia detected (Csokai, 2009). After the death of sick rabbits, their pathological-anatomical autopsy was performed and tissue samples of the kidneys, liver, lungs and brain (cortex and medulla, hemisphere of the brain and cerebellum) were taken. The tissue samples were fixed in 10 % solution of neutral formalin and histological preparations were produced by conventional method, they were stained with hematoxylin-eosin and examined by light microscopy (microscope Leica DM-2500, camera Leica DFC 450c, software Leica Application Suite Version 4.4., oc. 10, lens 100) (Garcia, 2002).

The obtained digital material was processed statistically with MS Excel 2007 computer program with the arithmetic mean definition (M), statistical *error* of the arithmetic mean (m). Probability difference (p) between the arithmetic mean of two variation series was determined by means of the probability measure ment criterion and in accordance with Student's tables (td). The difference between the two values was considered probable with equal probability $p \leq 0,05$.

Investigation was conducted in accordance with the «General ethical principles of experiments on animals» (Ukraine, 2001) and in compliance with the international

requirements of the European Convention «On protection of vertebrate animals used for experimental and other scientific purposes " (Strasbourg, 1985).

RESULTS

Epizootic situation concerning encephalitozoonosis in farm-raised rabbits.

According to the research findings, encephalitozoonosis of rabbits was registered on rabbit farms of Ukraine in 2014–2016. In rabbits affected by encephalitozoonosis pathogen asymptomatic, acute, and chronic course of disease was observed.

Asymptomatic course of encephalitozoonosis in rabbits is the most common. No clinical signs were observed in the infested rabbits. At the same time, these animals remain parasite carriers. Thus, a serological study showed that in 88 blood samples 30 of them had positive reaction to encephalitozoonosis pathogen, which accounted for 34 % and were indicative of parasitosis.

Acute course of the disease in rabbits manifested itself much less. In some animals the disease developed suddenly with a rapidly increasing symptom complex. The prodromal period of encephalitozoonosis in rabbits varied from several hours to 3 weeks. In our opinion, such a state depended on the immune system of their organism. Sometimes, during our research, acute disease course in rabbits was observed under the influence of stress factors. The main factors were transportation, re-grouping and other technological moments including temperature fluctuations of the environment.

Thus clinical symptoms occurred among all the age groups. However, the symptoms were sometimes poorly pronounced and depended on the fact which organs were affected in the first place (table 1).

Table 1. Epizootic situation concerning encephalitozoonosis in rabbits on the research farms

Farms	Number of animals	System of organism	Number of infested animals	Age, sex	Total number of sick animals	EI, %		
A.V.Datsiuk's farm	835	Nervous system	8	Young stock (1,5-3 months old)	10	1,19		
			2	Adult females				
			-	Adult males				
		Urogenital system	3	Young stock (1,5-3 months old)	4	0,48		
			1	Adult females				
			-	Adult males				
		Affection of organs of vision	1	Young stock (1,5-3 months old)	6	0,72		
			5	Adult females				
			-	Adult males				
		AF Slobodian	1365	Nervous system	11	Young stock (1,5-3 months old)	16	1,17
					4	Adult females		
					1	Adult males		
Urogenital system	5			Young stock (1,5-3 months old)	7	0,51		
	2			Adult females				
	-			Adult males				
affection of organs of vision	-			Young stock (1,5-3 months)	3	0,22		
	3			Adult females				
	-			Adult males				

In the study of 58 sick rabbits over 1–2 weeks, clinical symptoms were observed, with periodic aggravation of symptoms and the appearance of new morbid afflictions of the organism systems (nervous, *urogenital*, and organs of vision). Thus in 37 rabbits neurological

symptoms were observed which most often manifested themselves and amounted to 64 %, among them were found 28 young rabbits aged 1,5–3 months which accounted for 76 %. Defeat of the nervous system mostly manifested itself as vestibular disorders and in the first place was characterized by distortion of the neck in 37 rabbits (hence the popular name – «wryneck»). A head tilt to the right or left side and circus movements were observed in the sick rabbits (Fig. 1).



Fig. 1. Vestibular disorders in rabbits suffering from encephalitozoonosis

Thus, in the first day of illness, the general state and appetite in rabbits were not affected. Gradually, at 7-20 days, neurological symptoms progressed. Rotational movement around the axis of the body and ataxia appeared in most rabbits. Also, convulsions, paresis of one or two limbs, head tremors, shaking or nodding in the state of repose were noted in 32 rabbits. Nystagmus was observed in three rabbits. The state of those rabbits deteriorated. Later they developed a paresis of the pelvic limbs against which muscle degeneration quickly progressed. The animals ceased to eat food and drink water. Tonic-clonic seizures set in. The rabbits fell into a comatose state lasting up to 24–48 hours. 32 animals died while in a comatose state.

However, in 5 adult rabbits only a distortion of the neck was observed. Their general state was satisfactory at that. They did not lose appetite, ate and drank well. Over two or three months the above said clinical symptoms of the disease disappeared. Upon recovery female rabbits were capable of mating and bearing offspring.

Morbid affection of the urinary system in the sick rabbits, in the early stages of the disease, took a subclinical course. During this period disorders were insignificant and clinical symptoms were not typical – drowsiness, loss of appetite, which gradually led to anorexia, loss of weight, and dehydration were observed in 11 rabbits. According to our observations,

during the 4-6 week of disease, polyuria, polydipsia and urinary incontinence, leading to azotemia developed in rabbits, which was confirmed by biochemical blood analysis. Such animals were noticeable during clinical survey. They were exhausted, had expressed anemia visible mucous membranes; the genital area was constantly contaminated with urine, which later developed skin maceration. At 6-8 in week 10 rabbits developed acute interstitial nephritis, which manifested itself by a sharp deterioration of general state in animals. Body temperature increased to 40,5° C, polyuria and haematuria were evident. At 5-7 day oliguria or anuria developed, which progressed into acute renal failure. In 10 sick rabbits a state of coma and death were observed, which amounted to 91 % among rabbits with defeat of the urogenital system In one rabbit chronic interstitial nephritis, which later led to chronic renal failure was detected.

Defeat of the organs of vision in rabbits suffering from encephalitozoonosis is an important diagnostic feature. The symptoms manifested themselves within 5-10 days after the defeat of the central nervous system. Thus, in 10 rabbits with defeat of organs of vision, neurological symptoms were also observed in eight rabbits. The skin around the eyes was hyperemic, severe swelling of the eyelids was visible. Iridocyclitis was observed in five rabbits.

In the dynamics inflammation expanded to the choroid of the eye and affected all its parts causing panuveitis in 6 of 8 sick rabbits, which amounted to 75% (typical clinical symptom of encephalitozoonosis).

In two 1.5 month old rabbits, born by different females, reproduction of *E. cuniculi* was observed during fetal infestation in the lens of the eye, as manifested by accumulation of white mass. Thus, inflammation was observed in one eye only.

In nine sick rabbits which survived regression the disease took a chronic course. The clinical symptoms of the acute course observed by us for two to three months disappeared. The completion period of encephalitozoonosis was characterized either by recovery or death of the animals. Out of 58 animals suffering from encephalitozoonosis death occurred in 49, which amounted to 84 %. Death was caused by severe pathological changes developed as a result of action produced by *E. cuniculi* on the organism of the animals and complications due to layers of pathogenic microorganisms.

Enzymatic analysis of encephalitozoonosis. According to the analysis of blood samples from 88 rabbits it was found that 30 blood serums were positive (table 2). Thus, infestation extensiveness observed on Breeder A. V. Datsiuk's rabbit breeding farm determined by ELISA test method in 2014 amounted to 34 %. When analyzing data of the

table it becomes evident that in the groups of animals with ELISA test negative results optical density performance fluctuates within 0,66–2,55, privative of antibodies to *E. cuniculi*. While in the groups of animals with ELISA test positive results these values fluctuated within 3,45–400,00, indicating wide variations in immune responses. Based on the antibody titers the animals were divided into five groups.

Table 2. Investigation of blood serum in rabbits by ELISA test method for presence of specific antibodies to *E. cuniculi* Breeder A. V. Datsiuk's rabbit farm

Groups based on ELISA test results	n = 88	Average value of optical density units (M±m)	Boundary value of optical density units (Lim)
Reacting negatively	44	1,89±0,65	0,66–2,55
Seropositive, with low antibody titers	12	4,97±1,47*	3,45–8,50
Seropositive, with medium antibody titers	10	16,00±6,14*	10,50–28,75
Seropositive, with high antibody titers	4	162,72±58,00*	103,88–242,40
Seropositive, with high antibody titers and clinical signs	4	391,50±8,10*	381,00–400,00

Notes: *p<0,001 –compared to reference group

According to the research it was established that clinically healthy rabbits had different antibody titers, from low – 14 %, to high – 9 %. In our opinion, this is indicative of occult parasitosis.

It was also noted that the disease with pronounced neurological signs was accompanied by high antibody titers which approximated the value of 400 (absorbance units). these results show transition of the rabbit's organism in from the state of parasitosis to the state of disease with the following clinical manifestations of encephalitozoonosis.

Pathological-anatomical and histological investigation. With the death or slaughter of the sick rabbits, characteristic pathological-anatomical changes became evident. The animal corpses showed exhaustion. Mucous membranes as a rule were anemic. Characteristic morbid affectation was observed only in the areas of intensive parasitism of the pathogen.

As a result of the histological examination of the sagittal slices of the brain of the rabbits' corpses morphological changes were found in the pia mater, brainstem, less so in the cerebellum. Pia mater was affected almost in all the corpses, while inflammatory changes were well visible. Other parts of the brain showed significant loosening of the connective tissue fibers of the pia mater and permeability of serous exudate, rich in lymphocytes, erythrocytes, leukocytes, indicating the development of sero-hemorrhagic leptomeningitis. In the areas of significant affliction the pia mater showed marked changes of the brain substance.

Practically in all the areas of the brain hyperemia, per vascular and pericelaredema was detected by using light-optical techniques. Per vascular infiltration with focal inflammatory infiltrate was observed in places in the brain substance. In these areas, the substance in or near the cells, showed small oval spores of *E. cuniculi* ranging 1-2 micron in size. Scattered clusters of small oval spores of the parasite could also be seen in these areas of the brain substance. Thus, serous-hemorrhagic leptomeningitis and focal no purulent encephalitis developed in the brain of the sick rabbits which we believe was caused by reproduction of the encephalitozoonosis pathogen spores.

Through microscopic investigation the degree of severity of microstructural changes in the kidneys of different animals varied. In the kidneys of some rabbits, in which the course of the disease was acute, swelling of the epithelium of the convoluted tubule against the background of congestion, and vascular congestion of the intermediary zone and glomerular capillary loops were detected. Due to swelling of the nephrothelium, tubular lumen is narrowed in places, with lumen patency disrupted. In addition, in some rabbits the interstitium around the glomeruli was infiltrated in places with cellular elements including lymphocytes, histiocytes, and plasma cells, i.e. the formation of small globo-cellularinfiltrate was traced indicating the development of acute interstitial nephritis. In the kidneys of other rabbits, where the course of the disease was chronic, the lumen of the proximal tubules was significantly distended. In the epitheliocytes of the tubules significantly pronounced degenerative processes and morphologic alterations of cells were observed. However, interstitial thickening was traced in places between the tubules caused by the extensive growth of the connective tissue. Morbid affection of the tubules is clearly seen to be ranging from minor focal degeneration to extensive necrosis of epithelial cells, atrophy and destruction of the tubules. It should be noted that in the slightly enlarged lumen of some straight tubules eosinophilic accumulation of liquid is viewed, with small oval spores which are likely to be easily evacuated with urine.

Investigation showed no pathological changes in the preparations of the liver and lungs.

DISCUSSION

In recent years there have been significant changes in the structure of the pathogens of invasive and infectious diseases of farm animals.

Close international economic relations, intensive migration lead to changes of the habitat of certain parasitic and infectious diseases.

In such circumstances, to effectively combat and prevent their occurrence one has to carefully study the regional characteristics of the pathogen, its distribution and characteristics of the epizootic process. However, in the available reference sources we found no data on encephalitozoonosis in rabbits under Ukraine-specific conditions.

Through investigation of the epizootic situation of encephalitozoonosis in Ukraine, distribution and seasonality of the disease was established. According to the research, encephalitozoonosis in rabbits is recorded both on specialized rabbit farms and in private households.

It was established that infestation in rabbits occurs throughout the calendar year. The data received are confirmed by the investigation conducted in the European countries, indicating a year-round detection of rabbits suffering from encephalitozoonosis. In private households infestation was more often registered in autumn and winter period compared to spring and summer. One may assume that seasonal fluctuation of infestation is linked to the seasonal resistance of the organism and regrouping of the animals. Our research results are consistent with the data obtained in England, USA, and Austria. It should be noted that morbid affection caused by the encephalitozoonosis pathogen is encountered more frequently in rabbits, aged 1.5 to 3 months and rarely in reproductive animals.

Success in preventing and combating encephalitozoonosis in rabbits depends not only on deep knowledge of the local epizootology, timely diagnosis is also of importance. One of the important methods for diagnosis of the encephalitozoonosis complex is analysis of clinical signs. In the available literature there are many reports on the clinical course of encephalitozoonosis in rabbits. These reports were contributed at different times by scientists from Switzerland, Germany, Czech Republic, England, and Austria.

When studying the clinical manifestations of encephalitozoonosis it was established that prodrome in rabbits varied from several hours to 3 weeks. The course of invasion took asymptomatic, acute, or chronic forms. Key clinical symptoms were observed among all the age groups. Sometimes the signs were mild and depended on which organs were affected

in the first place. Asymptomatic course of encephalitozoonosis in rabbits was the most common. In rabbits suffering from encephalitozoonosis most likely to be affected are the brain, organs of vision and kidneys, as a result of parasite multiplication in these organs. Clinical symptoms, if they occur, are fairly typical. For making a diagnosis one should take into account the three main symptom complexes. Defeat of the nervous system is an often observed symptom.

Today, according to many scientists, serological investigation is the most important method for diagnosing encephalitozoonosis in animals. According to analysis of blood samples taken from 88 rabbits, 30 serums were found to be positive. In the groups of animals with negative ELISA test results, absorption indices fluctuated within 0,66-2,55 OOH, indicating the absence of antibodies to *E. cuniculi*. While in the groups of animals with positive ELISA test results these indices fluctuated within 3,4-400,0, indicating wide variations in immune response. According to our research it was established that clinically healthy rabbits had different antibody titers, from low – 14 %, to high – 9 %. In our opinion, this indicates occult parasitosis.

It was also noted that the disease with pronounced neurological signs was accompanied by high antibody titers which approximated the 400 (absorbance units) value. These results indicate a transition of the rabbit's organism from the state of parasitosis to disease with the ensuing clinical indications of encephalitozoonosis.

One of the important methods for diagnosing encephalitozoonosis in rabbits remains the method of morphological and histological investigation.

Following death or slaughter of the sick rabbits, characteristic pathological anatomical changes showed. Serous-hemorrhagic leptomeningitis and focal non-purulent encephalitis developed in the brain of the sick rabbits; we believe it was caused by multiplication of the spores of the encephalitozoonosis pathogen. The obtained data are confirmed by the research conducted by Csokai J., Cox J., who described *morbid* affliction of the brain caused by encephalitozoonosis.

According to scientists from Turkey and USA, microscopic investigations help establish that severity of microstructural changes in the kidneys of different animals may vary. Thus, in the kidneys and brain in rabbits suffering from encephalitozoonosis, structural-functional complex disorders developed as a result of parasite reproduction. The above described disorder is characteristic of encephalitozoonosis and can be used for establishing a final diagnosis.

CONCLUSIONS

The paper summarizes the results of our research on diagnostic methods and epizootology of encephalitozoonosis in rabbits on some farms of Ukraine. Data on the extensiveness of invasion based on the the results of the ELISA test method is submitted. An insight is given into clinical manifestation, pathological-anatomical and histological changes of some organs in the rabbits infested by microsporia spores. Encephalitozoonosis in rabbits was recorded on some farms of Ukraine. This rabbit disease is observed throughout the year. The least infested rabbit population is found in the spring and summer, extensiveness of invasion amounted to 0,63–0,79 %. A gradual increase in the number of the sick animals is noted in autumn with extensiveness of invasion up to 0,97 %. Maximum extensiveness of invasion (3,08 %) was registered in the winter season. Encephalitozoonosis was most often observed in young rabbits aged 1,5 months, extensiveness of invasion reached 0,65–11,76 %. In adult females extensiveness of invasion amounted to 0,32–6,9 %, in males– 0,16–3,85 % manifesting itself enzootic ally. Encephalitozoonosis in rabbits occurs asymptotically, acutely and chronically. Asymptomatic course of encephalitozoonosis in rabbits is the most common. There exist three symptom complexes: neurological disorders (tilt of the head, tremor of the head, curvature of the neck, rolling over along the longitudinal axis of the body, pelvic limbs paresis, ataxia, dystaxia, periodic agitation, tetanic seizures); *morbid affliction* of the organs of sight (conjunctivitis, iridocyclitis, paralysis of muscles of the eyeball, panuveitis, phaco plastic uveitis); morbid affection of the urinary system (enuresis, polyuria, polydipsia, acute or chronic interstitial nephritis).

As a result of ELISA test analysis it is established, that extensiveness of invasion in rabbits suffering from encephalitozoonosis is 34 %. Among the rabbits with positive reaction, groups with low ($4,97 \pm 1,47$ absorbance units), medium ($16,00 \pm 6,14$ absorbance units), and high ($162,72 \pm 58,00$ absorbance units) titer and clinically sick animals whose level of antibodies was the highest ($391,50 \pm 8,10$ absorbance units) were singled out.

Histological investigations have proven that serosanguineous leptomeningitis and focal suppurative encephalitis develop in the brain of rabbits. In the kidneys of rabbits during the acute course of the disease dystrophic-necrobiotic changes are observed in the system of tubules against the backdrop of congestion, during the chronic course - dilatation of the proximal convoluted tubules, atrophy of the epithelial cells of the tubules, focal tubular necrosis and vacuole degeneration and desquamation of the epithelium of the direct tubules

with moderate proliferation of the connective tissue in the interstices. The detected changes in the organs are caused by multiplication of the parasites in the form of spores.

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