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First molecular evidence of ocular transmission of Encephalitozoonosis during the intrauterine period in rabbits

Ozcan Ozkan[a,*], Alper Karagoz[b], Nadir Kocak[c]

[a] Çankırı Karatekin University, Faculty of Science, Department of Biology, 18100 Çankırı, Turkey
[b] Usak University, Faculty of Medicine, Department of Molecular Biology and Genetic, 64000 Usak, Turkey
[c] Selcuk University, Faculty of Medicine, Department of Medical Genetic, 42130 Konya, Turkey

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ABSTRACT

Many reports have been published on the suspected vertical transmission of *Encephalitozoon cuniculi*; however, prior to 2003, these reports were based on circumstantial evidence, such as histopathological, immunohistochemical, or serological diagnosis of the infection. In 2003, vertical transmission of the parasite was confirmed by detection of *E. cuniculi* DNA in fetuses with the nested polymerase chain reaction (PCR) technique. However, the passage of the parasite to eyes of fetus during the intrauterine stage still requires verification. In the current study, natively infected with parasite spores female rabbits were mated with non-infected males. All resulting offspring that died before ten postpartum days were investigated using molecular techniques to confirm the intrauterine transmission of the parasite to the offspring' eyes.

In total, 119 DNA samples from rabbit offspring tissues were collected from blood, kidney, brain, eye (both eyes were used as single samples), lung, placenta, liver and heart were used for PCR. Parasitic DNA in the eyes of offspring was detected (54%) 6 of 11 naturally seropositive mother rabbits. PCR results were found to be positive for the eyes of 63% (19/30) of the offsprings from seropositive rabbits. Therefore, mother rabbits naturally infected with *E. cuniculi* showed the molecular presence of the parasite in their offspring' eyes. Sequence analysis confirmed the partial DNA sequence data of *E. cuniculi* and blast analysis identified the agent as genotype I. These results confirm transmission of *E. cuniculi* to rabbit offspring' eyes in the intrauterine period. This is the first molecular evidence to show ocular transmission of the infection via an intrauterine route in rabbits.

1. Introduction

Encephalitozoon cuniculi is a Gram-positive, obligate, intracellular pathogenic parasite that is widely distributed and can cause latent disease, especially in lagomorphs. The disease was first described as encephalomyelitis causing motor paralysis in young rabbits [1,2]. Since that time, the use of molecular tools has identified four genetic strains of *E. cuniculi*. The strain I is mainly isolated from rabbits, strain II primarily affects rodents, strain III infection has been described in dogs, and a novel strain IV (human strain) has been reported in a human patient who underwent a kidney transplantation [3,4]. The rabbit and dog strains have also been isolated from humans; consequently, this opportunistic pathogenic parasite has been accepted as a zoonotic protozoon [5] and is therefore of both veterinary and public health importance [6].

In general, infection with *E. cuniculi* could occur via horizontal or vertical routes. The horizontal transmission process typically involves transmission of the spores of the protozoon by ingestion of food or water contaminated with infected urine and faeces. Another possible route of transmission is inhalation of spores originating from the same sources [7]. In rabbits, vertical transmission via an intrauterine (transplacental) route has also been reported from mother to fetus [8–10]. Infection of the eye with the parasite is believed to occur by this intrauterine route. The clinical ocular symptoms indicate the lens as the most affected part, even though this is an avascular, segregated compartment and the epithelial cells are surrounded by a thick capsule. However, during embryologic development, the lens capsule is thin and strongly vascularised; therefore, *E. cuniculi* can invade the eye after intrauterine transmission [9,11–13].

The clinical symptoms of the parasite infection can be quite varied among individuals in the later life stages. Although *E. cuniculi* infections are subclinical, most neurological, renal and ocular, findings have been reported in seropositive rabbits, respectively. These findings can also occur individually or in combination [7,14]. The ocular lesion is not a

* Corresponding author.
E-mail address: ozcanozkan@karatekin.edu.tr (O. Ozkan).

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newly emerging finding, as the first well-documented bilateral cataract due to *E. cuniculi* was described as an incidental occurrence in a laboratory rabbit in 1976 [9]. The typical lens lesion is unilateral, although bilateral phacoclastic uveitis has sometimes been reported by various authors, particularly in young rabbits [9,15–17]. Many studies have reported that vertical transmission can occur in various animal species; however, controversies still remain in the scientific literature. To the best of our knowledge, no molecular study has yet shown that the fetus eye can be infected with *E. cuniculi* during the intrauterine period.

The aim of this study, in naturally infected rabbits, was to confirm with molecular analysis to the transmission of *E. cuniculi* parasite to eyes of offsprings during intrauterine development.

2. Materials and methods

2.1. Animals

In Turkey (39° 55′ 31.9188″ N and 32° 51′ 58.6332″ E), the study, New Zealand white rabbits of both sexes, with body weights of 2.5–3.0 kg, were breed in a licenced commercial laboratory rabbit breeding facility. The animals were kept individually in cages in controlled housing conditions (temperature-19 ± 2 °C, humidity-50 ± 5% and lighting-12:12 h cycle). The rabbits were fed a standard commercial pellet diet and provided with fresh water ad libitum. Although the animals were not use any experimental procedure, all veterinary clinical practices were carried out in accordance with National Animal Ethical Regulations. Eleven naturally infected seropositive and one non-infected seronegative female rabbits were mated with six seronegative male rabbits in accordance with the routine breeding programme. The births of the animals were followed according to the mating schedule.

The mother rabbits sometimes cannot care about their offspring after birth, they can die. According to goal of this study, for this reason, eyes of offspring up to 10 days were used to avoid potential contamination of eyes because offspring' eyes were closed for ten days. For this reason, after birth, the vitality of the offspring was regularly examined four times a day for up to ten days. During observation, in case of dead offsprings were found and were removed from nest and were enrolled with their mother's identification number and stored at −20 °C until analysed. The plan of the study summarized in a graphical diagram as shown Fig. 1.

2.2. Blood samples and serology

Routine health monitoring of the rabbit breeding colony for *E. cuniculi* infection was conducted by collecting blood samples from a marginal ear vein of each animal for serological testing before the introduction of the rabbits in the colony. The sera were separated and stored at −20 °C until the serological analyses. The *E. cuniculi* specific antibody responses were determined in the rabbits using an ELISA kit (XpressBio, Frederick-USA) containing positive and negative controls (rabbit serum), according to the manufacturer's instructions. The sample was evaluated as positive in case the difference between the sample optical density (OD) and the negative control OD (Δ) was greater than or equal to 0.300.

2.3. Tissue sampling and DNA extraction

In the light of animal ethical principles, this study was planned only on dead offsprings and no applied any cesarean section. In this context, the sampling was optimised to avoid contamination and prevent transmission from one offspring to another and from one tissue to another within the same offspring. For DNA extraction, some of tissues samples such as lungs, brains, livers, kidneys, hearts were taken from of dead offsprings and placenta samples were also taken from died

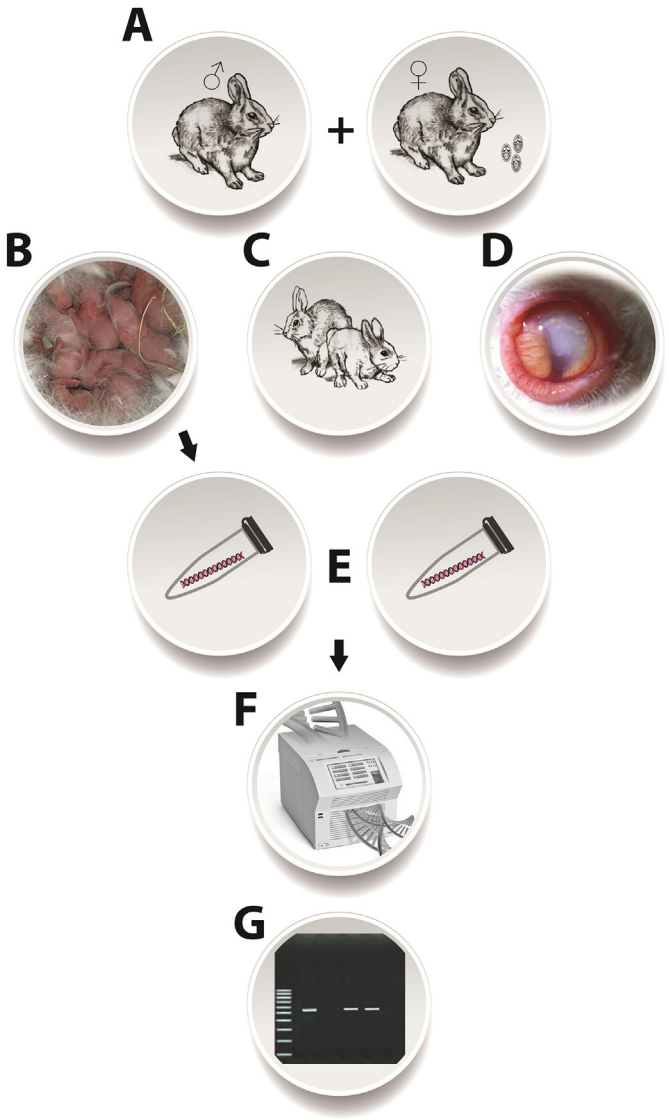

Fig. 1. Naturally infected with *Encephalitozoon cuniculi* spores seropositive female rabbits were mated with seronegative male animals (A). Decease during or after birth, the nest of animals was routinely monitored for ten days (B). In young rabbits (C), ocular lesions with a visible white mass can cause (D). In current work, dead offspring animals used for DNA extraction. DNA samples obtained from some organs and especially eyes (E). At next step, performed PCR amplification (F). Later, whether intrauterine ocular passage of the parasite was present (G) using the molecular technique was investigated.

immediately after birth but the eyes were taken from all the offspring since this study focused on showing the passage of the parasite to the eye in the intrauterine period. Therefore, both eyes were carefully removed intact from the dead offsprings and stored in separate containers as single samples for later DNA extraction. The positive control samples were obtained from the Department of Biology and Genetics, University of Veterinary Medicine in Kosice, Slovakia. The DNA extraction procedures for the spores and tissue specimens were run in parallel. The specimens (each 25 mg) were homogenised with ceramic beads in phosphate buffered saline solution under sterile conditions. Subsequently, to disruption of the spores, 200 µL of the resulting suspension was exposed to mechanical microwave (600 W) three times for 20 s each [18]. At this stage, the DNA was extracted by incubating the specimens in tissue lysis buffer containing 25 µL proteinase K (25 mg/mL) for 1 h at 56 °C. The samples were then processed according to the manufacturer's instructions (Qiagen, Hilden, Germany). As the next step

of DNA purification, the DNA was dissolved in sterile water for use in PCR.

2.4. PCR amplification and DNA sequencing

PCR amplification protocol was performed, as described previously by Valencakova et al. (2005). The specific primer pairs ECUNF and ECUNR were used for the amplification of a 550-base pair (bp) small subunit ribosomal RNA product. The DNA obtained with the PCR reaction was analysed by electrophoresis in 1% agarose gel; the gel was then stained with ethidium bromide and gel images were photographed. The PCR amplicons were sequenced according to the manufacturer's instructions using the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 3700 Genetic Analyser (Applied Biosystems). The results of sequence analysis were also used to confirm the DNA sequence data of the protozoon using the Basic Local Alignment Search Tool software (BLAST).

3. Results

The serological screening results were used to group the animals according to their serostatus. In the current study, 11 seropositive and 1 seronegative female rabbits and 6 seronegative male animals were mated, for a total 18 rabbits used for breeding. The one seronegative female was evaluated as controls. By ten days, 30 dead offsprings from 11 seropositive females and one dead offspring from seronegative female were obtained after birth. In total, 119 DNA samples from rabbit offspring tissues were collected from blood (n = 25), kidney (n = 24), brain (n = 24), eye (n = 31 both eyes were used as single samples), lung (n = 9), placenta (n = 7), liver (n = 2) and heart (n = 2) were used for PCR.

The parasite DNA was detected in the offsprings' eye 6 (54%) of 11 seropositive mother rabbits. In the present work, PCR product results revealed the eyes of the offsprings of 63% (19/30) seropositive female rabbits (n = 11) were positive but the other tissue samples were negative as shown Table 1. The sizes of fragments of the PCR products were compared using a standard 100 bp DNA ladder. Electrophoresis samples (Fig. 2) showed an amplified product of 550 bp from *E. cuniculi*, as summarized in Table 1, thereby confirming the passage of *E. cuniculi* to the fetus eye during the intrauterine stage in rabbits. Database homology searching was performed with BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Besides, the parasite sequences data (ECUNTR 1–7) were matched with *E. cuniculi* (GenBank accession numbers AL590444.1; KC513606.1; Z19563; HM04949.1; L13295; LO7255; NM-001041130.1; L13332; L17072; L29560) according to BLASTn analysis and defined as genotype I. Our sequence data blasted 100% homology with accession numbers AL590444.1 and KC513606.1.

4. Discussion

Encephalitozoon cuniculi infection has been reported at various rates in many veterinary medicine studies that have employed different diagnostic tools and numerous sample sizes and hosts [6,19]. Specific antibodies develop within 21 days post-infection. In rabbits, in previous studies reported that the seropositivity rate for *E. cuniculi* infection was higher than 50% in various countries [6,20]. The most common horizontal route of the infection in rabbits begins in the intestine and after several of replication in the intestinal epithelium, where infective spores are disseminated throughout the body the heart, lungs, liver, and spleen. At this stage, after the parasite reaches equilibrium with immune response of animal, *E. cuniculi* spores can reside in kidneys, brain and eyes, without ever causing clinical signs in animal. Nevertheless, when the equilibrium of immune was broken, the main lesions occur as neurological, renal and ocular forms of the infection, consequently, clinical signs are observed in rabbits [7,14,20]. The disease has been diagnosed using different methodologies, such as microscopical, histopathological, serological and molecular techniques so far [7,20]. Nonetheless, this infection is not easily diagnosed in living animals because its symptoms are subclinical. However, the chronic form of the disease is characterised by neurological, renal or ocular forms in which one or a combination of clinical forms can be seen [7,20]. Conversely, many physical ophthalmological examinations of ocular cases in rabbits have revealed a visible white mass in the eye, uveitis and cataracts [14–17]. The source of the ocular lesions due to *E. cuniculi* has been presumed to be an infected mother [9,12,13]. During the first trimester period of gestation in rabbit embryological development, the lens placode forms followed a few days later by formation of the lens capsule; therefore, the parasite is assumed to enter the lens at this. The spores can also be trapped in the anterior lens capsule stage [11–13]. The rabbit offsprings have sealed eyes and do not open their eyes before postpartum day ten. Postpartum transmission in rabbits often occurs within 6 weeks from an infected animal [7,21]. For this reason, in our study, only offspring that died up to 10 days postpartum were used to avoid possible environmental contamination of their eyes.

Various reports of suspected intrauterine transmission of *E. cuniculi* infection have been published, but all have been based on circumstantial evidence, such as the histopathological, immunohistochemical or serological diagnosis of *E. cuniculi* infection [17,20]. The route of contagion continues to be discussed in the veterinary literature, but only three significant publications have described vertical transmission from non-contaminant guinea pig pups and rabbit fetuses under gnotobiotic conditions and aseptic cesarean operations [8,10,22]. However, one of these studies examined pregnant rabbits infected with the parasite and conducted autopsies on the 28th day of gestation. Baneux and Pognan [10] were able to demonstrate a vertical transmission route for the parasite by using PCR to search for the presence of *E. cuniculi* DNA in some of the fetus tissues. However, the eye and the lens were not investigated by these authors, so the possibility of intrauterine infection of rabbit eyes remained in question. Banneux and Pognan [10] demonstrated the presence of the parasite in the brain, liver and kidney tissues of the offspring. In this study, as molecular technique, a qualitative PCR testing is used to showing presence or absence of the parasite DNA in tissue and results are expressed as negative or positive for *E.cuniculi* DNA. However, by contrast, in our study, no tissue other than the eye was infected. Although seropositive titers are not correlated with the degree of parasitism or clinical signs of the infection, the serologic evidence may be used to confirm exposure or infection status.

Table 1
Encephalitozoon cuniculi DNA detection in the samples of the offsprings.

Mother rabbits number	Dead offspring number	PCR result	Offspring tissues								Total
			Blood	Kidney	Eye	Lung	Placenta	Liver	Heart	NC	
Seropositive (n = 11)	31	(−)	24	22	24	11	8	6	2	2	113
		(+)	0	0	0	19	0	0	0	0	
Seronegative (n = 1)	1	(−)	1	1	1	1	1	1	0	0	6
Total (n = 12)	32		25	23	24	31	9	7	2	2	119

Negative control (NC); Positive (+); Negative (−).

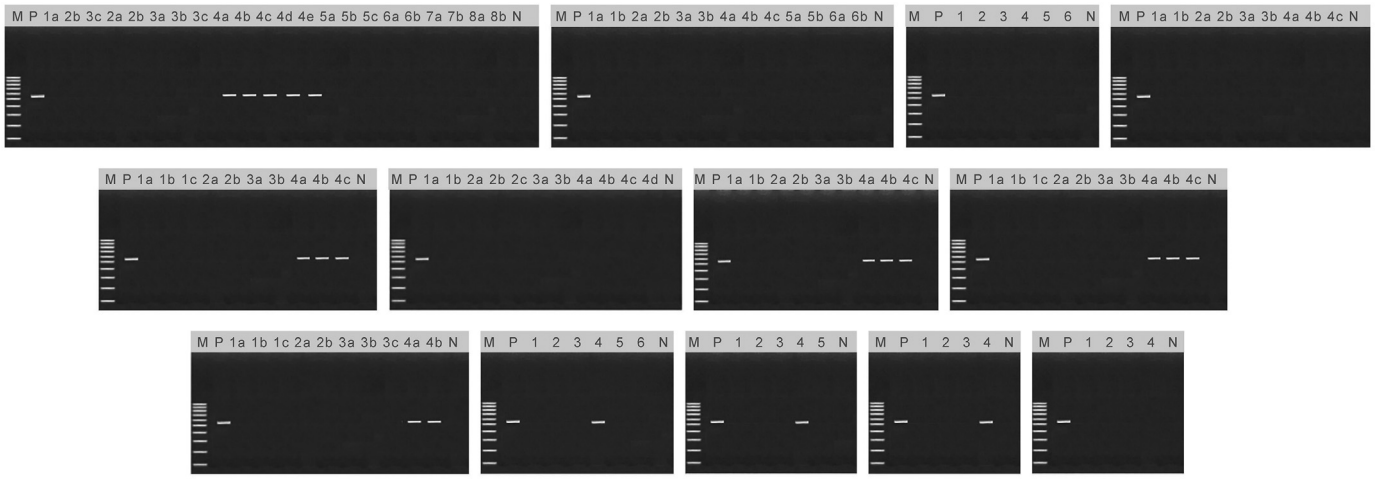

Fig. 2. Polymerase chain reaction was applied on tissue specimens of the offsprings using specific primer pairs. The samples were obtained amplicons approximately 550 bp. Each letter represents a offspring. In lines: Molecular weight (100 bp repeat) ladder (M); Positive *E. cuniculi* control (550 bp) (P); Blood (1); Kidney (2); Brain (3); Eye (4); Lung (5); Placenta (6); Liver (8); Heart; (N); Negative control.

In immune competent animals can develop antibodies against *E. cuniculi* spores for the life of the host; however, the seroconversion does not result in a protective response or immunity for the host, namely they do not offer protection against reinfection [7,19,20,23,24]. This might reflect differences in the duration and count of spores used for the exposure, condition of animal, environmental conditions, feeding, hygiene of facility, but the most important factor the immune response of the animal during gestation. In conclusion, the results of the current study clearly show that pregnant rabbits naturally infected with *E. cuniculi* produced dead offsprings (deceased during partum or after birth) that demonstrated the presence of parasite DNA, especially in their eyes. Therefore, this study provides the first molecular evidence confirming the ocular transmission of the genotype I parasite by an intrauterine route in rabbits.

Conflict of interest

We declare that we have no conflict of interest.

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