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The seroprevalence of *Encephalitozoon cuniculi* among healthy pet rabbits in Norway

Seroprevalens av *Encephalitozoon cuniculi* hos friske kjæledyrkaniner i Norge

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Table of contents

Abstract

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Summary: *Encephalitozoon cuniculi* is a spore-forming obligate intracellular parasite, belonging to the genus Microsporidia. It is zoonotic and can infect both humans and other species with immunocompromised individuals being especially susceptible. The pathogen is a source of disease in pet rabbits (*Oryctolagus cuniculus*) and targets several organs typically including the brain, eyes and kidneys. Clinical signs of encephalitozoonosis often observed include head tilt, ataxia, swaying, uveitis or polyuria, and severe infection may lead to death. Clinical diagnosis is challenging due to many differential diagnoses and the fact that *E. cuniculi* can give an asymptomatic chronic infection with antibody response. In this study serum samples from 105 clinically healthy pet rabbits from six counties in Southern Norway were analysed for IgG (Immunoglobulin G) and IgM (immunoglobulin M) antibodies against *E. cuniculi* by an Immunofluorescent antibody test (IFAT). The results revealed the antibody prevalence to be 59% (62/105). In regard to specific antibodies, 5% (5/105) had elevations in IgM antibodies only, 38% (40/105) in IgG antibodies only and 16% (17/105) had elevations in both IgG and IgM antibodies. No risk factors such as gender, diet, living conditions or access to direct sunlight showed correlation with being infected with *E. cuniculi.* There were no obvious geopgraphical differences in seroprevalence. The findings of this study showed *E. cuniculi* to be a widespread pathogen in the healthy rabbit population in Southern Norway.

Definitions and abbreviations

E. cuniculi	*Encephalitozoon cuniculi*
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFAT	Immunofluorescent antibody test
Antibody titre	The highest serum sample dilution showing fluorescence in the IFAT testing modality

Introduction

Rabbit keeping in Norway

In Norway, rabbits are increasingly kept as pets, making them the third most common pet after dogs and cats (Buseth, 2013). This is combined with a change in the way owners view this species, with increasing focus on their biological requirements and welfare (Buseth, 2013). This shift is shown through the fact that more people keep rabbits freely indoors, or in adequately sized enclosures combined with a heated house outdoors (Buseth, 2013). According to the Norwegian Rabbit Association, rabbits thrive best when kept in pairs or groups, as they are highly social animals. The Norwegian Rabbit Association therefore recommends against keeping rabbits alone - a recommendation that is being heeded by more and more owners. How the general transition from single- to multi-rabbit households will impact on transmission of infectious diseases, is unknown. *Encephalitozoon cuniculi* (*E. cuniculi*) is an infectious pathogen in pet rabbits (*Oryctolagus cuniculus*) shown to be widespread in several countries (Dipineto, et al., 2008) (Harcourt-Brown & Holloway, 2003) (Kunzel & Joachim, 2010). The aim of the present study was to determine the seroprevalence of antibodies against *E. cuniculi* in healthy pet rabbits in Norway.

Encephalitozoon cuniculi

General

Encephalitozoon cuniculi is a spore-forming obligate intracellular parasite, belonging to the genus Microsporidia (Orenstein, et al., 2005) (Didier, et al., 1995). Its taxonomic positioning has been discussed (Kunzel & Joachim, 2010), with some arguing that microsporidia should be classified together with fungi due to their similar anatomical structures, i.e. the polar tube (or filament) (Latney, et al., 2014) (Kunzel & Joachim, 2010). *Encephalitozoon cuniculi* was previously described as a basic eukaryote, due to the lack of mitochondria (Kunzel & Joachim, 2010). However, after the discovery of mitosomes, a very small and reduced form of mitochondria, *E. cuniculi* was rather compared to Microsporidia within the fungi phylogenetic tree, where they are thought to be positioned between the ascomycete and basidiomycete clade (Kunzel & Joachim, 2010). There are multiple species within the genus *Encephalitozoon*, several of which are opportunistic pathogens in immunocompromised humans.

Primarily rabbits are affected by *E. cuniculi* but the pathogen can also be found in other species such as mice, rats, hamsters, guinea pigs, muskrats, sheep, goats, pigs, horses, domestic dogs, domestic cats, foxes, and non-human primates (Didier, et al., 1995). (Levkutova, et al., 2004). *E. cuniculi* has been shown to infect humans that have acquired immunodeficiency syndrome (AIDS) or that are otherwise immunocompromised. Common symptoms seen in humans with encephalitozoonosis include fever, diarrhoea, polyuria and keratoconjunctivitis (Varga, 2014).

Life cycle

The entire life cycle of *E. cuniculi* takes 3 to 5 weeks to complete (Richardson, 2000). The host is infected horizontally by ingestion or inhalation of spores, usually via contaminated food or water, or vertically by transplacental transmission (Weiss, 2001). Once in the bloodstream or lymphatic system, the spores are disseminated through the body via macrophages/monocytes (Didier, et al., 2009) and soon reach their target organs such as the brain, kidneys and liver (Varga, 2014). Little is known about how *E. cuniculi* can survive and replicate within the macrophages. A study by Didier, et al (2009) suggests that *E. cuniculi* spores may inhibit apoptosis to prolong their survival rate.

Once in their target organs, the spore germinates, a 'polar-filament' extrudes from the polar cap, and this filament is thought to help gain access into the host cell (Varga, 2014). When the spore's polar-filament has penetrated the host cell, the spore passes its sporoplasm and nucleus to the host cell through the polar-filament. Following this, the sporoplasm divides, thus creating meronts. The meronts then undergo several replications by binary fission. The meronts first convert to sporonts, and the sporonts further convert to sporoblasts. Sporoblasts synthesize new spores so the host cells rupture, releasing mature spores with polar filaments (Jordan, et al., 2006). The mature spores measure about 2.5 x 1.5 μm and are oval in shape (Gjerde, 2011). Their thick capsule is strongly Gram-negative, which is a characteristic feature (Varga, 2014). The spores are then excreted in urine and faeces, or are transmitted transplacentally to offspring (Mathis, et al., 2005). The life cycle of *E. cuniculi* is illustrated in figure 1.

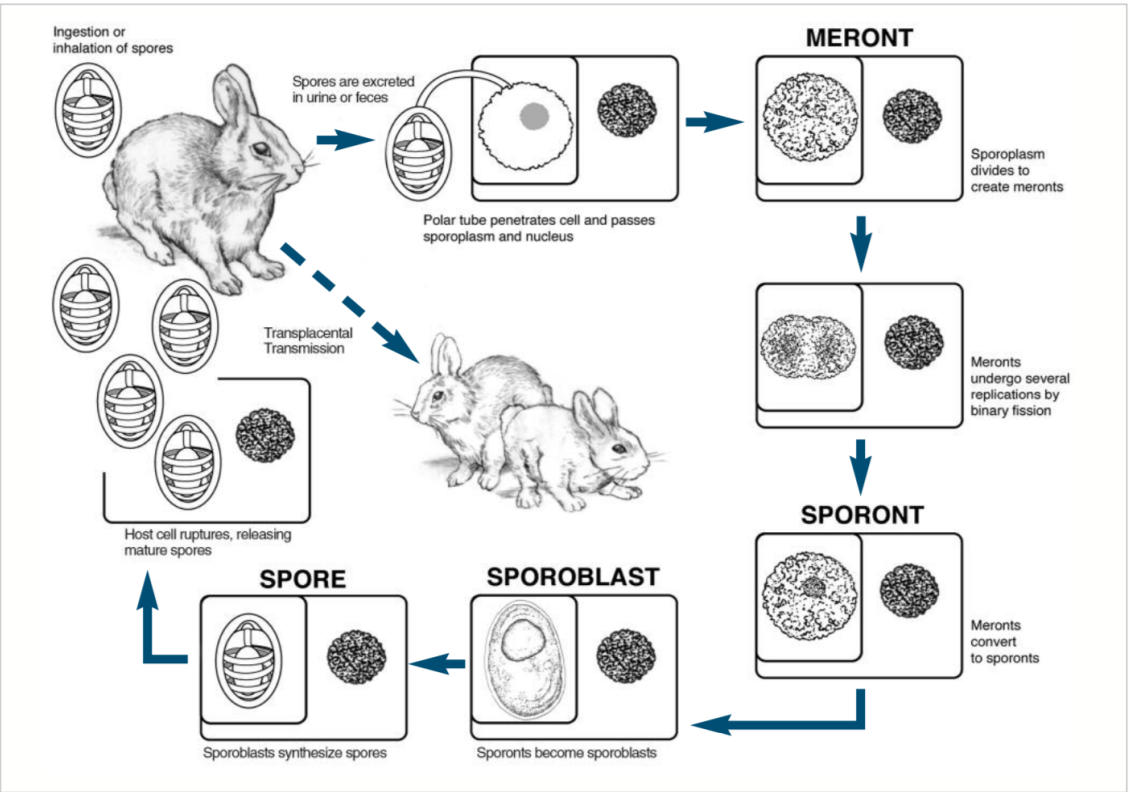

Figure 1: Life cycle of *Encephalitozoon cuniculi* (Jordan, et al., 2006)

E. cuniculi-spores can remain viable in the environment for at least 4 weeks at room temperature (Varga, 2014). There are four genotypes of *E. cuniculi*, genotype I being responsible for infections in rabbits, whilst genotypes II, III, and IV predominantly infect rodents, dogs and humans, respectively (Didier, et al., 1995). The genotypes can be differentiated by molecular methods, such as PCR and DNA sequencing, with target genes including the internal transcribed spacer region of the ribosomal RNA gene (Hinney, et al., 2016)

Symptoms

Most infections of rabbits with *E. cuniculi* are subclinical (Jordan, et al., 2006). In symptomatic rabbits, clinical signs associated with encephalitozoonosis are broadly grouped into three categories based on the predilection sites of *E. cuniculi*; central nervous system, renal, and ocular (Varga, 2014). The neurological symptoms include vestibular disease, head tilt, seizures, ataxia and posterior paresis in the acute phase, and ‘swaying’ when resting, stargazing (eyes directed upwards as the head is involuntarily bent backwards), aggression, deafness, blindness, incontinence, loss of balance, and uneaten caecotrophs in the chronic

phase (Varga, 2014). Caecotrophs are a normal part of a rabbit's digestive system and are re-ingested to enhance nutrient absorption. They are consumed directly from the anus, and uneaten caecotrophs can be a sign of general illness (Harcourt-Brown, 2005). Signs of renal disease include polydipsia and polyuria, urinary incontinence, mild renal insufficiency, and chronic renal failure (Varga, 2014). Lens rupture, uveitis, secondary hypopyon, cataracts, and blindness are the ocular signs associated with encephalitozoonosis (Varga, 2014).

Once *E. cuniculi* has infected the host, it will eventually cause granulomatous lesions in the brain (focal non-suppurative granulomatous meningoencephalitis) and interstitial nephritis (focal to segmental) in the kidneys, even in asymptomatic infections (Varga, 2014) (Cox & Gallichio, 1978). This chronic granulomatous inflammation is thought to be responsible for the clinical signs caused by *E. cuniculi* (Varga, 2014). The granulomatous response is thought to be triggered by the rupture of host cells and release of foreign material (Varga, 2014). The clinical signs that develop are therefore associated with granulomatous nephritis, granulomatous encephalitis, vestibular syndrome and chronic renal failure (Varga, 2014). Rabbits can, as previously mentioned, develop lens rupture, pyogranulomatous uveitis and cataracts (Varga, 2014). It has also been shown that when clinical signs are observed in a pet rabbit with encephalitozoonosis, they usually are neurological (Jordan, et al., 2006).

Immunology

Both the cell-mediated and the humoral immune response is involved in the response to infection with *E. cuniculi* (Varga, 2014), but the humoral antibody response is the main focus of this study. The humoral immune response is activated in the body upon recognition of an infective agent. Upon first exposure to an infective agent such as *E. cuniculi*, the body's native B-cells will migrate from the lymphoid tissue, become activated, and begin to proliferate into plasma cells. The plasma cells will then produce antibodies specific to the antigen (Abbas, 2015). The initial response usually takes 5-10 days and is dominated by the production of immunoglobulin M (IgM), although some immunoglobulin G (IgG) may also be produced at this stage (Abbas, 2015). An illustration of antibody production can be found in figure 2. IgM levels tend to reach a peak 17 days post-infection (Jeklova, et al., 2010). The reported duration of IgM antibodies in the blood varies, from 5 weeks (Jeklova, et al., 2010) to 6-9 weeks (Rich, 2010). Repeat or chronic infection leads to the production of antibodies dominated by IgG (Abbas, 2015). Since a chronic infection with *E. cuniculi* often occurs, high

levels of IgG may be present at all times with or without reinfection (Kunzel & Joachim, 2010).

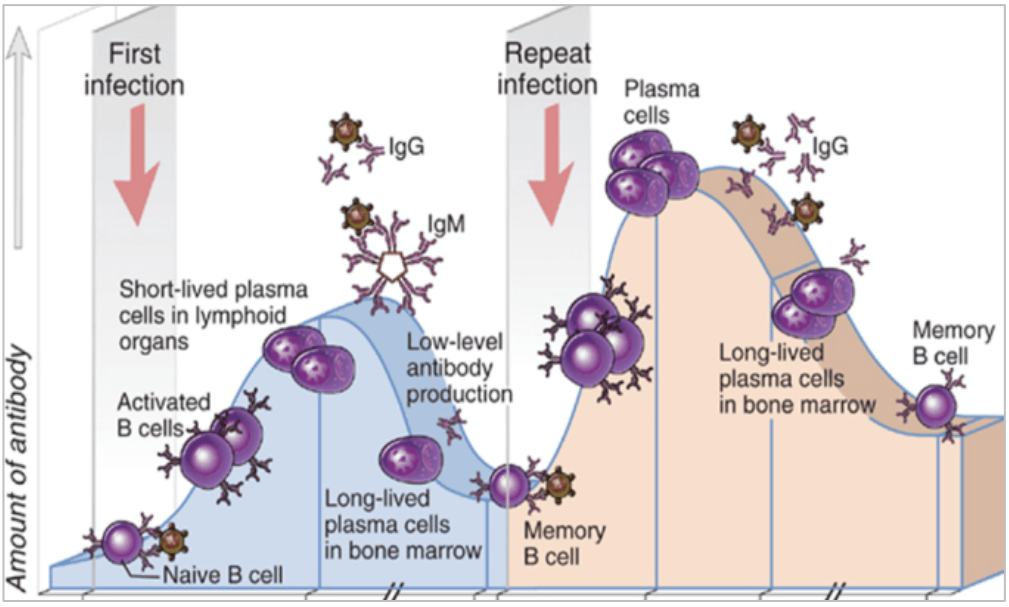

Figure 2: Antibody production after infection (Abbas, 2015)

Differential diagnosis

Due to the many non-specific clinical signs displayed in rabbits with encephalitozoonosis, the list of differential diagnoses is often long. Some symptoms may be more "classical" than others, and for these, infection with *E. cuniculi* may be higher up the list of differential diagnoses. Encephalitozoonosis may also be secondary to another disease or stressful event in a rabbit, and thus the underlying cause of disease may need to be treated in order to control the *E. cuniculi* infection (Kunzel & Joachim, 2010). Central nervous system (CNS) impairment can be caused by a variety of pathogens, from *Pasteurella multocida*, *Listeria monocytogenes*, *Escherichia coli* and *Pseudomonas aeruginosa*, to, less commonly, infections with *Toxoplasma gondii,* Human Herpesvirus-1 or rabies (Latney, et al., 2014). Non-infectious differentials could be neoplasia (e.g. lymphosarcoma), toxicities (e.g., lead toxicity), conditions such as hydrocephalus, infarcts in both brain and spinal cord, and vertebral pathology, like scoliosis or fractures (Latney, et al., 2014). Rabbits with encephalitozoonosis are commonly presented with vestibular disease. However, the same clinical sign can be caused by a number of other causes. Otitis media/interna, usually caused by *P. multocida*, can cause a peripheral vestibular disease (Kunzel & Joachim, 2010) (Latney, et al., 2014). Meningoencephalitis, enterotoxaemia and sepsis may cause central vestibular

disease, as well as other conditions of the brain previously mentioned (Latney, et al., 2014). Hepatic encephalopathy may also be a cause of neurological impairment (Latney, et al., 2014). With regards to the ocular symptoms associated with *E. cuniculi*, these can be compared to other kinds of uveitis like bacterial and lens-induced uveitis and cataracts in geriatric patients (Latney, et al., 2014) (Kunzel & Joachim, 2010). Table 1 shows a summary of important differential diagnoses to encephalitozoonosis as presented in a study by Latney, et al. (2014).

Clinical presentation	Infectious differentials	Noninfectious differentials
CNS impairment	*Pasteurella multocida*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Listeria moncytogenes* Uncommon: Human Herpesvirus-1, *Toxoplasma gondii*, rabies, and *Baylisascaris procyonis*	Lymphosarcoma, lead toxicity, hydrocephalus, cerebral infarcts, spinal infarcts, vertebral scoliosis, and vertebral fractures
Central vestibular disease	Bacterial, viral, protozoal, and verminous meningioencephalitis, Enterotoxemia, sepsis	Lead toxicity, metastatic neoplasia, osteomyelitis of the petrous temporal bone, cerebral infarcts, and hepatic encephalopathy
Ocular disease	Bacterial uveitis (unilateral disease is uncommon)	Lens-induced uveitis caused by geriatric cataract formation

Table 1: Common differential diagnoses to encephalitozoonosis in rabbits (Latney, et al., 2014)

Diagnostic testing

A number of different diagnostic tests have been utilised for the diagnosis of *E. cuniculi* in rabbits. These can be grouped into: i) tests that aim to detect the organism itself, or components thereof, such as detection of spores in the urine or faeces generally using molecular techniques like PCR, and ii) tests that aim to detect the host response to infection, such as serological tests for IgM or IgG antibodies. There are also a number of non-specific, but supportive, tests that can be performed, such as biochemistry and haematology. Direct detection of spores in urine and faeces using PCR or microscopy has shown to be unreliable due to sporadic excretion (Rich, 2010) (Jeklova, et al., 2010). Serological testing is therefore the most commonly used testing modality in clinical practice. However, appropriate interpretation of serology requires an understanding of the dynamics of this pathogen within rabbit populations.

Serology

Indirect immunofluorescent antibody tests (IFAT), carbon immuno-assays (CIA) and enzyme-linked immunosorbent assays (ELISA) have been developed for detecting IgG and IgM in serum or plasma (Jeklova, et al., 2010). Using both IgG and IgM may help in defining the

stage of infection, as a rise in IgM titre is indicative of an acute infection or reinfection (Jeklova, et al., 2010). A positive IgM titre supports the theory of the rabbit being infected within the previous 35 days (Latney, et al., 2014). The IgG levels are expected to rise within 2-3 weeks of first infection (Latney, et al., 2014). However, even the presence of elevations in both IgG and IgM does not give a definitive diagnosis of encephalitozoonosis, as these findings can also be present in asymptomatic animals and animals with concurrent disease (Cray, et al., 2020) (Jeklova, et al., 2010). The same levels of IgG have been found in both asymptomatic as well as clinically ill rabbits (Csokai, et al., 2009). The titre may therefore not correlate with the degree of clinical symptoms (Kunzel & Joachim, 2010). In contrast, a negative test for IgG and IgM indicates that the rabbit has not been exposed to *E. cuniculi*, and is thus useful to eliminate encephalitozoonosis from the differential list (Cray, et al., 2020) (Kunzel & Joachim, 2010).

Immunofluorescent antibody test (IFAT)

The immunofluorescent antibody test (IFAT) is a method in which antibodies conjugated to a fluorescent dye are used to detect the presence of specific antigens (or another antibody) to which they bond. This can be both a direct and an indirect antibody reaction, depending on whether the fluorescent dye is attached to a primary or secondary antibody. The indirect method is illustrated in figure 3. These antibodies, together with their fluorescent conjugates, are then visualised through a fluorescent microscope. Specifically, the template is prepared with *E. cuniculi* antigens, and then the serum or plasma of a patient is added to the template. If antibodies towards *E. cuniculi* are present in the sample, they will bind to the *E. cuniculi* antigen. A secondary antibody towards rabbit *E. cuniculi*-antibodies (essentially an anti-antibody, that binds to an antibody as it would to an antigen) that has been conjugated with a fluorescent dye is then added. The template is then washed to remove any free antibodies. Where binding occurs, the antigen-antibody-anti-antibody complex will be visible

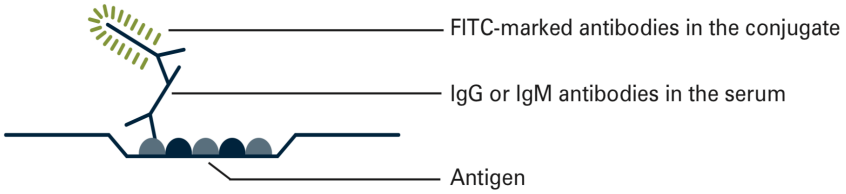

Figure 3: Illustration of an indirect binding of antibodies to an antigen (Megacor Diagnostics GmbH)

by fluorescence microscopy. These steps are repeated in different dilutions of the serum, so that a cut-off value can be set according to the degree of fluorescence in each of the dilutions. IFAT is a semi-quantitative antibody test, such that the quantity of antibodies present in a sample can be classified to some extent. Illustration of the IFAT method can be found in figure 4.

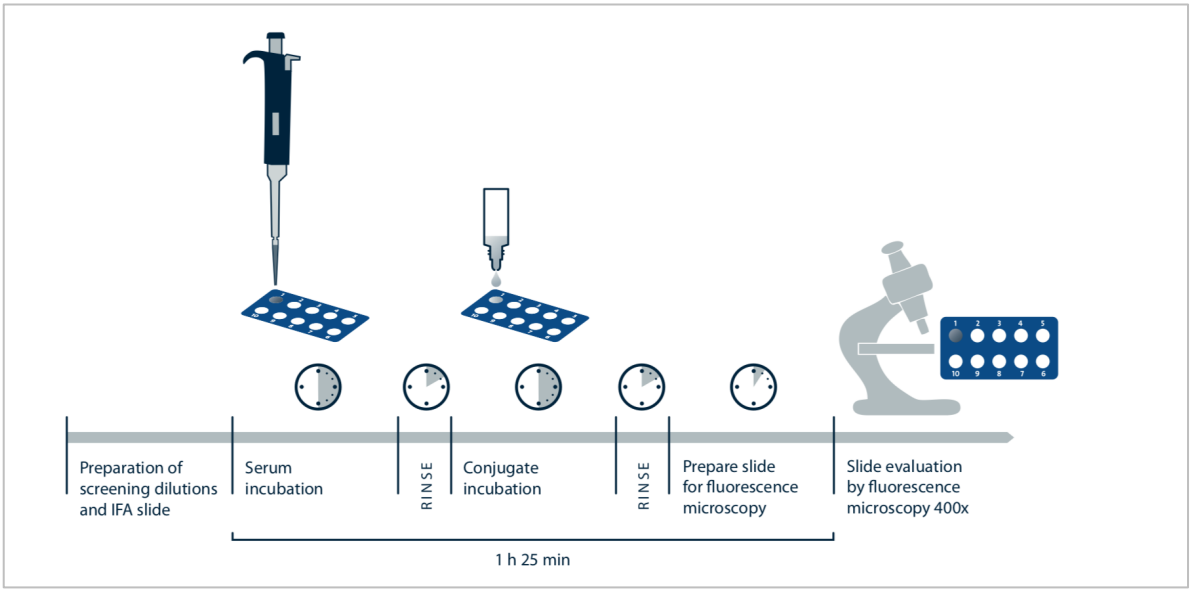

Figure 4: IFAT method (Megacor Diagnostics GmbH)

Non-specific testing

Haematology, blood biochemistry and serum electrophoresis may be useful in the suspected case of encephalitozoonosis, but cannot be used to set a definitive diagnosis (Kunzel & Joachim, 2010). Blood parameters of interest include albumin, alfa-, beta- and gamma-globulin, and the albumin to globulin ratio (A/G ratio). In one study, rabbits with suspected encephalitozoonosis had a lower A/G ratio and elevated levels of gamma globulins than clinically normal rabbits (Rich, 2010). Since *E. cuniculi* infections often lead to a chronic inflammatory process, a rise in the gamma globulins are expected (Rich, 2010). Other biochemical parameters of interest include potassium, phosphorus, urea and creatinine, according to different organ pathology (Jeklova, et al., 2010). Urea and creatinine levels may be especially useful in confirming the stage of renal affection and thus long-term prognosis. In haematology, changes in neutrophil and lymphocyte count may be expected (Jeklova, et

al., 2010). Urine analysis may also be of use to evaluate kidney function further and to rule out concurrent urinary tract disease (Kunzel & Joachim, 2010).

Post-mortem examination

As a method of confirming a cause of death, post-mortem investigation of renal tissue showed difficulty locating *E. cuniculi* spores due to fibrotic or granulomatous reaction (Rich, 2010). Low amounts of organisms have been found in histological investigation of cerebral lesions (Rich, 2010) as well as in tubuli lumens in the kidneys (Csokai, et al., 2009). Microscopically, *E. cuniculi* spores can be stained with Giemsa-, Gram- or Goodpasture-carbol fuchsin stains, and less effectively with haematoxylin or eosin (Suckow, et al., 2002). Typical pathological lesions in the kidneys are pinpoint white colonies or grey indented areas on the renal cortical surface (Suckow, et al., 2002). In the central nervous system, granulomatous encephalitis can be characteristically observed, as well as lesions of the spinal cord (Suckow, et al., 2002). PCR of liquified lens material and/or brain tissue can also give a definitive diagnosis (Csokai, et al., 2009). However, histological examination with special staining has been shown to be the most sensitive post-mortem test (Csokai, et al., 2009).

Treatment and prognosis

Treatments that have been used for infections with *E. cuniculi* are generally aimed at reducing the spore proliferation and migration, reducing inflammation caused by the rupture of host cells and controlling neurological problems, as well as more general supportive care and addressing secondary conditions (Latney, et al., 2014). The use of fenbendazole for treating encephalitozoonosis in rabbits has been evaluated in two studies, with best effect shown with a dosage of 20 mg/kg daily for 28 consecutive days (Suter, et al., 2001) (Sieg, et al., 2012). Non-steroid anti-inflammatory drugs (NSAIDs) may also be considered, e.g. meloxicam. The effects of NSAIDs have to be evaluated against the risk of reported NSAID-associated nephrotoxicity since the kidneys may already have reduced function due to pathology from *E. cuniculi*. The use of steroids to reduce inflammation by ruptured host cells has been practiced (Latney, et al., 2014). However, the risk of suppressing the immune system and exposing the rabbit to further infection and damage may outweigh the benefits of treatment (Latney, et al., 2014). Renal disease, ocular disease or other symptoms may also need separate treatment according to the degree of pathological changes. Diazepam or midazolam may be used for seizures (Latney, et al., 2014).

The prognosis after treatment varies and may depend upon the severity of clinical signs (Kunzel & Joachim, 2010). In particular, the degree of neurological affection may indicate the prognosis, as rabbits presenting with severe rolling/seizures have been reported to have a lower success rate of treatment (Kunzel & Joachim, 2010). Sieg et al. (2012) reported that rabbits treated with fenbendazole were 1.6 times more likely to survive past day 10 than rabbits left untreated. Suter et. al. (2001) has reported a preliminary study on 16 rabbits presenting with neurological signs after being clinically infected with *E. cuniculi* and showing IgG titres of 1:640 serum dilutions or higher being treated with fenbendazole orally every day for four weeks. The outcome showed that 50% of rabbits recovered completely, whilst 13% had a persisting mild head tilt after treatment. In the study 31% (5/16) of the rabbits were euthanized due to poor response to treatment (Suter, et al., 2001).

The aim of the study

The aim of our study was to determine the seroprevalence of antibodies to *E. cuniculi* in healthy pet rabbits in Norway. The results are intended to provide a valuable tool for the interpretation of serological results amongst the Norwegian pet rabbit population, and thus aid veterinarians in the diagnosis of encephalitozoonosis. Furthermore, we also wished to explore whether there are any particular risk factors associated with *E. cuniculi* infection in Norwegian rabbits. Risk factors of interest included living conditions like multiple rabbit households, drinking water from a bowl, indoor- versus outdoor rabbits, age and gender. This is interesting in regard to the route of infection, and whether vertical transmission during pregnancy is more common than horizontal transmission later in life.

Methods and materials

Animals

The study population consisted of 105 clinically healthy pet rabbits (45 females, 60 males) from six different counties in Norway (Figure 5). Health status was determined by the examining veterinarian, based upon both clinical examination on the day of sampling as well as information from the rabbit owner. The age of the rabbits ranged from 5 months to 9 years old. Living conditions varied from caged all the time, to free some of the day, to free roaming all day and the study population included animals kept both indoors and outdoors. Diets varied, although all rabbits in the study population were fed hay on a daily basis.

The rabbits were presented for a health check at NMBU Small Animal Clinic (n = 72) as well as three private veterinary clinics in Oslo (n = 3), Bergen (n = 26) and Skien (n = 4). The rabbits from NMBU were there exclusively to participate in a study on normal blood parameters in pet rabbits, whilst the rabbits from other clinics were chosen when visiting clinics for routine procedures (i.e. health checks, neutering, etc.). Inclusion criteria were the absence of any clinical signs observed in a full physical examination, with particular attention to the neurological and ocular examination. Health status and the absence of symptoms were determined by the examining veterinarian, based upon both clinical examination on the day of sampling as well as the owners reporting their rabbit to be healthy at the time of sampling.

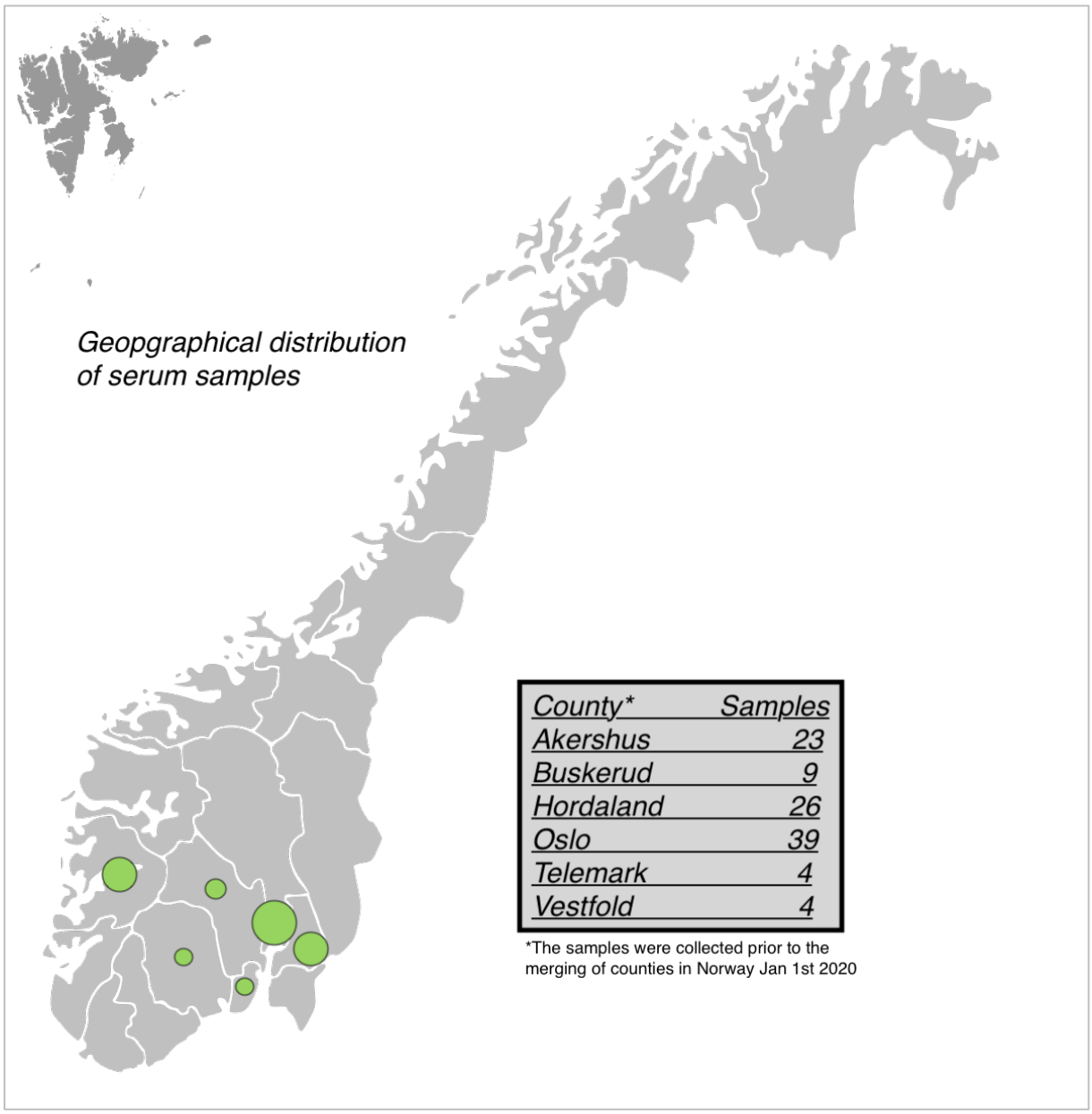

Figure 5: Geographical distribution of 105 pet rabbits included in a study of seroprevalence of *Encephalitozoon cuniculi* among healthy pet rabbits in Norway

The physical examination included examining eyes, ears, mouth cavity and teeth, examination of palpable lymph nodes, thoracic auscultations, palpation of the abdomen, examination of the external reproductive organs and extremities, judging the skin- and fur quality, body condition scoring and measuring temperature. Owners provided information on eating and drinking habits, defecation and urination, living conditions and their general impression of overall health. Additional information on the specific contents of the health checks can be found in Appendix 1 and 2. Only one veterinarian at each clinic examined the rabbits, and each was considered to be experienced in exotic pet medicine.

Sampling

Venous blood was collected from the lateral saphenous vein. In the samples taken at NMBU Small Animal Clinic, a 2.5 ml Terumo syringe with a Genia disoject 23G x 25mm needle was used. After collection, the blood was gently added into a vacutainer (Vacuette Tube, 3 ml clot activator) following removal of the vacutainer top to prevent haemolysis due to rapid emptying. After centrifugation (5500 rpm for 10 minutes), the serum from each rabbit was put into a mini-collect tube (Greiner Bio-one Gmb, 0,25-0,5 ml), and frozen at -18°C. Serum samples were stored at -18°C until shipping as a single batch to the diagnostic laboratory (Laboklin GmbH, Austria) in August 2019.

Detection of antibodies - IFAT

An indirect semiquantitative immunofluorescent antibody test (IFAT) was performed on all samples using a commercial kit (MegaFLUO *Encephalitozoon cuniculi* IgG and IgM, by Megacor GmbH). The principle of the test is explained in the introduction and illustrated in figure 3 and 4. Dilutions used were 1:80, 1:320, 1:640, 1:1240, 1:1280 and 1:2560. The cut-off value is set at 1:80 by Laboklin, such that samples with no fluorescence at a dilution of 1:80 are considered negative, whilst samples showing fluorescence at dilutions higher than 1:80 are considered positive. Samples showing fluorescence only at the starting dilution of 1:80 are considered positive for the purpose of this study, although the laboratory terms these 'borderline'. The antibody titre is defined as the last serum dilution showing fluorescence.

Questionnaires

One questionnaire from NMBU (Appendix 3) and another from the private clinics (Appendix 4) was designed to obtain information about the age, gender, housing, feeding, and general health condition of the rabbits, and was provided to the 105 rabbit owners to fill in and return. 86 out of 105 rabbit owners returned the questionnaires. A second questionnaire was distributed by email to the owners of those rabbits with elevated IgM and requested information on the presence of clinical symptoms of *E. cuniculi* infection after the date of sampling. (Appendix 5). Nine of thirteen rabbit owners answered this questionnaire.

Statistical analysis

Pearson's chi-square test for independence was used to see whether the distribution of rabbits testing positive for *E. cuniculi* was significantly associated with various potential risk factors.

The variables included gender (female or male), if they were spayed/neutered, whether the rabbits were fed hay, muesli (a bran/seed mixture intended for rabbit feed) and green leafy vegetables, if they were living with other rabbits, drinking water from a bowl, and if they had frequent access to direct sunlight or not. All questions (except gender) were answered yes or no, and the factors and seropositivity were plotted into a 2x2 chi-square table for independence (Table 2). In all cases, a p-value was calculated using an online p-value calculator (*https://www.socscistatistics.com/tests/chisquare/default2.aspx*). A p-value of <0.05 indicates a statistically significant association between having antibodies against *E. cuniculi* and the risk factor. These analyses were based on the returned questionnaires from the rabbit owners (Appendix 3 and 4)

	Seropositive	Seronegative	Total
Risk factor - yes	x	y	x+y
Risk factor - no	a	b	a+b
Total	x+a	y+b	86

Table 2: Example of 2x2 table used to calculate the p-value of potential risk factors in healthy pet rabbits seropositive for *Encephalitozoon cuniculi*.

Results

Antibody prevalence

Testing showed a prevalence of 59% (62/105) of healthy pet rabbits in Norway having IgG and/or IgM antibodies against *E. cuniculi* when borderline samples (serum dilution 1:80) were also considered positive. Among these, 40/105 rabbits (38%) had an elevation in IgG antibodies only, 5/105 rabbits (5%) had an elevation in IgM antibodies only and 17/105 rabbits (16%) had an elevation in both IgG and IgM antibodies (table 3). If borderline samples were considered negative, the total antibody prevalence would be 36/105 (34%). The IgG prevalence would be 23/105 (22%), IgM prevalence 2/105 (2%), and IgG and IgM prevalence 11/105 (10%) (table 3).

The prevalence varied some between the counties. Akershus showed a prevalence of 57% (13/23), Buskerud 44% (4/9), Hordaland 65% (17/26), Oslo 62% (24/39), Telemark 75% (3/4) and in Vestfold there were no seropositive rabbits (0/4). All calculations on the county percentages were done considering the borderline samples positive.

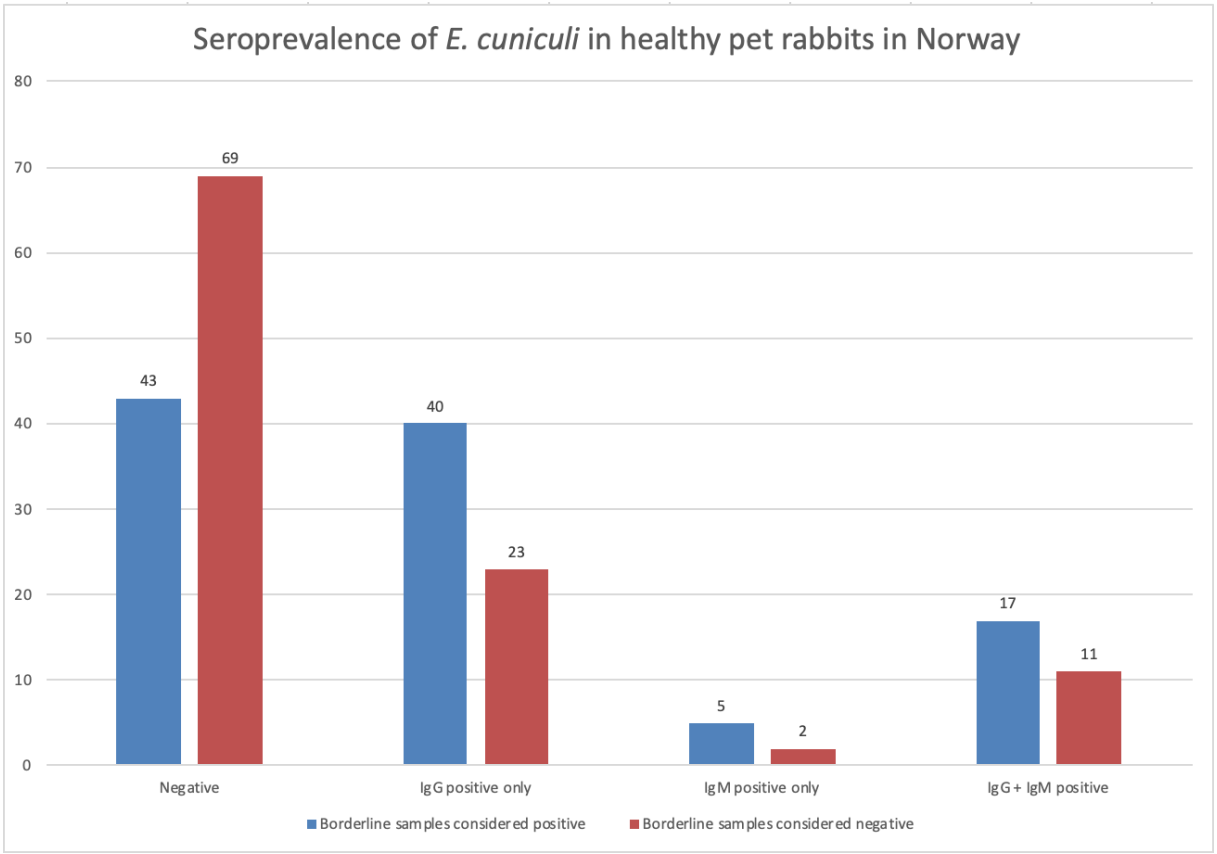

Table 3: Seroprevalence of IgG and IgM antibodies against *Encephalitozoon cuniculi* among healthy pet rabbits in Norway

The antibody titre categories ranged from <1:80 (considered negative) to 1:2560, the latter being the highest dilution showing fluorescence for IgG that is tested for in the laboratory, and 1:640 being the highest dilution showing fluorescence for IgM. See table 4 for distribution of antibody titres among the tested rabbits. Out of the 17 rabbits with elevated IgM and IgG antibodies, most of them also showed a high serum dilution for IgG, with 12/17 rabbits showing an IgG titre at 1:2560 dilution, 2/17 an IgG titre at 1:1280 dilution, 1/17 an IgG titre at 1:640 dilution, and only 1/17 rabbits only being positive for IgG at 1:80. The remaining 5 rabbits with elevation in IgM did not have elevated IgG. Of these 5 rabbits, 3 showed an IgM titre at 1:80 dilution, 1 at 1:320, and 1 at 1:640.

Antibody titre (Highest serum dilution showing fluorescence)	**IgG**	**IgM**
<1:80	48	83
1:80 (Borderline according to Laboklin definition)	23	9
1:320	6	6
1:640	2	7
1:1240	1	-
1:1280	4	-
1:2560	21	-
Total number of rabbits	105	105

Table 4: Distribution of serum dilutions of *Encephalitozoon cuniculi* IgG and IgM antibodies in healthy pet rabbits in Norway

Risk factors

Pearson's chi-square test did not indicate any of the risk factors investigated being associated with being antibody positive for *E. cuniculi*. All rabbits in this study were fed hay, so a chi-

square analysis of this factor is not possible. All 2x2 tables can be found in Appendix 6. P-values for all risk factors are presented in table 5.

Potential risk factor	**P-value**
Gender	0.22
Neutered/spayed	0.84
Multiple rabbits	0.66
Eating hay	-
Eating muesli	0.25
Eating plants/leafy greens	0.58
Water from bowl	0.96
Access to direct sunlight	0.23

Table 5: P-values from chi-square analysis of potential risk factors for healthy Norwegian pet rabbits having antibodies to *Encephalitozoon cuniculi*. P<0,05 indicates significance.

Individuals with elevated IgM - owner follow-up

Out of the 13 rabbit owners contacted by email for the follow-up investigation, four did not respond to the questionnaire. Of the nine who responded, seven rabbits had not shown any clinical signs of encephalitozoonosis during the 6-12 months between sampling (January-July 2019) and answering the questionnaire (December 2019). However, one rabbit had presented with polyuria and polydipsia to a local veterinary clinic and later recovered, and another rabbit had developed a weakness in the hindlimbs as well as polyuria and was euthanized due to poor quality of life. A necropsy was not performed so the cause of symptoms is only speculative and cannot be confirmed. These two rabbits showing clinical signs of encephalitozoonosis had IgM antibody titres at 1:320 and 1:640 dilutions, respectively. They also had elevations in IgG with titres at 1:1280 and 1:2560 dilutions.

Discussion

In this study, 59% of healthy pet rabbits in Norway had IgM and/or IgG antibodies against *E. cuniculi* (assuming borderline samples to be positive). This correlates with results from similar studies on rabbits in other countries. Asymptomatic rabbits have shown high rates (37-68%) of infection in many countries, including UK, Japan, and Italy (Kunzel & Joachim, 2010) (Harcourt-Brown & Holloway, 2003) (Dipineto, et al., 2008). The results in this study shows a higher seroprevalence than those reported in Korea (23%) and Nigeria (17%), similar to the UK (52%) and Japan (58%), and lower than Italy (68%) (Kunzel & Joachim, 2010) (Shin, et al., 2014) (Tee, et al., 2011) (Keeble & Shaw, 2006). Several of these studies use different testing modalities, with ELISA (Enzyme-linked immunosorbent assay) and CIA (Carbon-linked immunoassay) being the most frequently used. One study (Cray, et al., 2020) showed some difference in results when comparing the ELISA, CIA and IFAT in examining serum for *E. cuniculi* antibodies, so this has to be taken into consideration when comparing studies using different testing modalities. An overview of different *E. cuniculi* seroprevalence studies are presented in table 6.

Country	**Number of rabbits**	**Testing modality**	**Prevalence (%)**	**Reference**
Italy	47	ELISA, CIA	68	(Dipineto, et al., 2008)
Norway	105	IFAT	59	(Our study, 2020)
Japan	337	ELISA	58	(Tee, et al., 2011)
UK	97	ELISA	52	(Keeble & Shaw, 2006)
Korea	186	ELISA	23	(Shin, et al., 2014)
Germany	218	IFAT	18	(Hein, et al., 2014)
Nigeria	237	IFAT	17	(Okewole, 2008)
Sweden	106	CIA	7	(Eriksson, 2007)

Table 6: Seroprevalence of *Encephalitozoon cuniculi* in asymptomatic rabbits, and testing modality used in several countries.

A surprising find was the difference in seroprevalence between rabbits from Sweden and Norway, with Sweden showing a seroprevalence of 7% (Eriksson, 2007) versus the Norwegian seroprevalence of 59%. As the two neighbouring countries have quite similar

climates and geographical positioning one would expect the seroprevalence to be somewhat alike. One reason could be that the Swedish study included rabbits only from one Small Animal clinic in Stockholm. If rabbits from several parts of the country were included one may have seen more similar results. Furthermore, the Swedish study analysed the samples using the CIA method which may contribute to a variation in results compared to our study using the IFAT method (Cray, et al., 2020). Further research of interest could include performing a new Swedish study comparing antibody seroprevalence among rabbits from similar areas to the counties represented in our study to investigate whether this variation is legitimate or just a factor of the study design.

The seroprevalence is quite different if the borderline samples are considered negative (Table 3). Since the cut-off value was set by the laboratory analysing the samples, one may argue that another laboratory would put a different cut-off, thus giving a different picture of the seroprevalence. However, the laboratory has set this value due to nonspecific antigen binding which can occur at lower dilutions and give a false positive result (Chalupsky, et al., 1973). Furthermore, other similar studies have also performed the IFAT with cut-off values around the one used in this study (Okewole, 2008) (Hein, et al., 2014), although performed in other laboratories. In these studies samples were considered positive if they showed fluorescence at dilutions equal or higher than the cut-off value.

In regard to the geographical spread, there seems to be no significant findings regarding the seropositivity. One exception is Vestfold county, where none of the rabbits tested were seropositive. An important factor here could be that three out of the four rabbits tested in Vestfold came from the same household. The calculations also showed that the bigger groups seemed to be closer to the general Norwegian prevalence. The case may be that the smaller groups have not included enough individuals to give a good aspect on the general prevalence in that area. The information on geographical spread is also based upon the rabbit's current habitat and does not include information on where the rabbit originated from.

None of the studied risk factors showed a significant association with being infected with *E. cuniculi* using Pearson's chi-square test (table 4), meaning none of the factors give a higher likelihood of a rabbit having antibodies against *E. cuniculi.* This is also underlined in a study conducted by Keeble and Shaw in 2006. In their study they found no significant correlation

between a rabbit having antibodies against *E. cuniculi* and living conditions such as having access to an outside area and living with multiple other rabbits (Keeble & Shaw, 2006). This suggests that the main method of infection of rabbits with *E. cuniculi* may be by transplacental transfer of spores to litters during pregnancy, rather than horizontally later in life. If horizontal transfer was the main transmission route, it might be expected that rabbits living together would have a significantly higher seroprevalence than rabbits living alone. However, this was not the case in our study. Indeed, in our study, where rabbits were living together, individual rabbits could be seropositive, but others seronegative, indicating a lack of horizontal transfer. In an Italian study (Maestrini, et al., 2017), only rabbits from breeding colonies showed a significant association with being infected with *E. cuniculi*, which may indicate horizontal transmission. However, breeding rabbits from the same colony would be expected to descend from the same lines, so vertical transmission of spores from an infected ancestor could also have been the relevant factor. Furthermore, if horizontal transmission was the major route of infection, then rabbits roaming outside during parts of the day might be expected to be at greater risk of becoming infected due to exposure to *E. cuniculi* spores shed from wild animals such as mice or other rodents. However, as no such correlation was found, this suggests that, along with the high proportion of asymptomatic seropositive individuals as well as only sporadic excretion of spores in urine (Kunzel & Joachim, 2010), vertical transmission is more likely than horizontal transmission. Experiments and research to test this theory could provide more definitive evidence. One method of reducing the seroprevalence in the pet rabbit population, considering this theory were true, could be to recommend all breeding rabbits to go through testing for *E. cuniculi*, and only use the seronegative individuals in further breeding.

Our study showed a high number of asymptomatic rabbits with IgG antibody titres at high serum dilutions. Latney, et al. (2014) suggested that rabbits have a persistently high IgG titre due to chronic infection. Kunzel et.al. (2010) claims IgM could be a helpful marker for the stage of infection. However, in our study, rabbits showing no symptoms also had elevations in IgM antibodies. This suggests that rabbits can have an asymptomatic acute or reactivated infection. This is important in the matter of the infectiveness of the disease. An interesting question is whether these asymptomatic individuals might be excreting spores and thus providing a potential route of infection to others whilst not showing any symptoms themselves. It would also be interesting to know if these rabbits acquire long-term

pathological changes, suggesting an ongoing chronic infection. Persistently high IgG titres in asymptomatic animals may also suggest that the onset of clinical signs at a given time may be caused by stress, changes in environment or another concurrent disease that triggers an activation or increase in severity of an already present *E. cuniculi* infection within that individual. As such, a part of the clinical work-up should be to examine the environment, changes or causes of stress by underlying undiagnosed problems. Another question to consider is whether rabbits can have a rise in IgM antibodies upon reactivation of a latent infection, and not only upon new infection or reinfections. As the results of our study showed almost 20% of rabbits having elevated IgM, and with the theory of vertical transmission taken into consideration, one may suspect that these rabbits are frequently experiencing a reactivation of spores and thus experiencing elevations in IgM. There seems to be limited research on this matter.

Diagnosing encephalitozoonosis in rabbits may be challenging due to the many differential diagnoses and the variable symptoms (table 1). Furthermore, a positive test for *E. cuniculi* may be an incidental finding and not related to the cause of the symptoms presented; thus clinical relevance has to be considered (Varga, 2014). In many cases other possible diagnoses must be excluded, and sometimes response to treatment has to be considered (Kunzel & Joachim, 2010). Owner compliance and economy may also be a factor in diagnosing encephalitozoonosis. This study provides a general picture on the seroprevalence of *E. cuniculi* among healthy pet rabbits in Southern Norway. The results could be used as a tool by veterinarians to help informing owners about a potentially very serious and fatal disease. A known prevalence may also show both owners and veterinarians that this infection is widespread and aid in the diagnosis and rapid treatment to prevent rabbits succumbing to a potentially treatable illness.

Conclusion

In conclusion, 59% (62/105) of healthy pet rabbits in Norway demonstrated antibodies against *E. cuniculi* by serum analysis using an immunofluorescent antibody test. This correlates with similar studies done in other countries and shows a widespread infection among Norwegian pet rabbits. Rabbits showing no clinical signs of encephalitozoonosis had both IgM and IgG levels indicating an active and/or chronic infection. No risk factors showed any correlation with being infected with *E. cuniculi*, strengthening the theory of a transplacental route of infection being the most dominant. We would suggest that this could be a useful area of further investigation as if this was definitively shown to be the case, then measures could be implemented to reduce the infection prevalence in pet rabbits (e.g., recommended testing of rabbits to be used for breeding, such that only seronegative rabbits are in fact used for breeding).

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Appendices

Appendix 1a: Health check NMBU Small Animal Clinic - original

Referansenummer: _____ Dato: _____
Journalnummer: _____

Klinisk undersøkelse
Vekt: _____ BCS (1-5): _____ Temp (til slutt): _____
RR (først): _____ HR: _____ Slh: _____

	u.a.	Anmerkning
Ausk pul/cor:	□	
Holdning/ganglag:	□	
Øyne:	□	
Ører:	□	
Nese:	□	
Insp/palp incisiver/molarer:	□	
Hud/hårlag:	□	
Palpasjon abdomen:	□	
Androgenitalområde:	□	
Poter:	□	

Stressnivå: mild/moderat/kraftig

Helhetsvurdering:
_____

Appendix 1b: Health check NMBU Small Animal Clinic - English

Reference number: _____ Date: _____
Journal number: _____

Physical exam
Weight: _____ BCS (1-5): _____ Temp (at end of exam): _____
RR (start): _____ HR: _____ Mucous membranes: _____

	No remarks.	Note
Auscultation pul/cor:	□	
Posture/movement:	□	
Eyes:	□	
Ears:	□	
Nose:	□	
Insp/palp incisors/molars:	□	
Fur/skin:	□	
Abdomen palpation:	□	
Genital area:	□	
Paws:	□	

Stress level: mild/moderate/high

Total assessment:
_____

Appendix 2a: Health check other clinics - original

* Navn på kanin og eier: _____

* Dato for prøvetaking: _____

* Merking av prøve:
  Dato - Navn på kanin - Navn på eier - Utdelt klinikknummer

Ring rundt det som passer:

- Normalt aktivitetsnivå: JA / NEI
- BCS (Body condition score): 1 - 2 - 3 - 4 - 5
- Normal appetitt og drikkelyst: JA / NEI
- Normal avføring (farge, størrelse, mengde): JA / NEI
- Normal funksjon av bevegelsesapparatet: JA / NEI
- Fravær av ”head tilt” eller andre nevrologiske symptomer (sirkling, nystagmus, etc.): JA / NEI
- Fravær av tannsykdom ved visuell undersøkelse av munnhulen: JA / NEI
- Fravær av neseflod: JA / NEI
- Fravær av øyesykdommer (katarakt) ved generell undersøkelse: JA / NEI
- Står på faste medikamenter: JA / NEI .
- Hvis ”JA”, hvilke(t) medikament(er)?

Appendix 2b: Health check other clinics - English

* Name of rabbit and owner: _____

* Date of sampling: _____

* Sample markings:
  Date – Rabbit name – Owner name – Clinic number

Circle the suitable answer:

- Normal activity level: YES / NO
- BCS (Body condition score): 1 - 2 - 3 - 4 - 5
- Normal appetite and drinking: YES / NO
- Normal feces (colour, size, amount): YES / NO
- Normal function of locomotor system: YES / NO
- Abscense of ”head tilt” or other neurological symptoms (circling, nystagmus, etc.): YES / NO
- Abscense of dental disease upon inspection of mouth cavity: YES / NO
- Abscense of nasal discharge: YES / NO
- Abscense of ocular disease upon general inspection: YES / NO
- Administered regular medication: YES / NO .
- If ”YES”, which medical substance?

Appendix 3a: Owner information NMBU Small Animal Clinic - original

Eiers navn: _____Bosted(fylke): _____

Telefon: _____E-post: _____

Kaninens navn: _____Rase: _____

Fødselsdato: _____

Kjønn: _____Kastrert/ikke kastrert (Sett ring) Hvis ja, ved hvilken alder: _____

Har du oppfattet kaninen som frisk de siste 3 måneder? Ja/Nei (Sett ring).

Spiser og drikker kaninen som normalt? Ja/Nei

Har kaninen normal avføring? Ja/Nei

Bor kaninen med andre kaniner? Ja/Nei

Hvis ja, antall og kjønn, kastrert/ikke kastrert på den/de andre kaninene: _____

Kort beskrivelse om hvordan kaninen bor (ca areal, tilgang til gjemmesteder, type underlag, mulighet for å gnage/grave/løpe, soveplass osv):

_____

_____

_____

Er kaninen noen gang ute i direkte sollys? Ja/Nei

Hvis ja, hvor ofte og hvor lenge:

_____

Har kaninen noen gang vært syk: Ja/Nei

Hvis ja, gi en kort beskrivelse av sykdomsforløpet:

_____

Hva spiser kaninen? (Sett ring rundt det som passer). Høy Pellets Gnagermix Frukt Grønnsaker Bær

Evt. type frukt/grønnsaker/bær (ca mengde og hvor ofte):

_____

_____

_____

Annet:

_____

_____

Har kaninen noen gang tilgang til gress eller annen vegetasjon? Ja/Nei

Hvis ja, hva og hvor ofte?

_____

__

Hva drikker kaninen av? Skål/Flaske/Annet _____Står kaninen på noen medisiner? Ja/Nei

Er eller har du mistanke om at kaninen er drektig? Ja/Nei

Jeg samtykker med dette at prøveresultatene kan bli brukt til forskning og undervisning. Resultatene vil være anonymisert og eiers personinfo vil ikke bli gjengitt på noe tidspunkt.

Signatur: Dato:

_____

_____

Appendix 3b: Owner information NMBU Small Animal Clinic - English

Owner name: _____Residence (county): _____

Phone: _____Email: _____

Rabbit name: _____Breed: _____ Date of birth: _____

Gender: _____Neutered/intact (Sett ring) If yes, at what age: _____

Have you perceived the rabbit as healthy the last 3 months? Yes/No (Circle).
Does the rabbit have normal fecal pellets? Yes/No
Is the rabbit living with other rabbits? Yes/No
If yes, number and age, spayed/intact?

_____

Short description of living conditions (approx. area, access to hiding places, type of bedding, ability to dig/gnaw/run, sleeping spot etc.)

_____
_____
_____
_____

Does the rabbit have any access to direct sunlight? Yes/No
If yes, how often and for how long?

_____

Has the rabbit ever been ill? Yes/No
If yes, please give a short description of the disease process:

_____

What does the rabbit eat? (Circle the fitting). Hay Pellets Muesli Fruit Greens Berries
Type of fruit/greens (type, amount)_____

_____
_____

Other:

_____
_____

Does the rabbit ever have access to grass or other vegetation? Yes/No
If yes, what sort and how often?

_____
__

What does the rabbit drink from (bowl, bottle, other) _____
Is the rabbit receiving any medication? Yes/No
Is or do you suspect the rabbit to be pregnant? Yes/No

I consent to the test results being used for research and teaching purposes. The results will be anonymous, and owner's personal information will not be shared at any given time.
Signature: Date:

_____
_____

Appendix 4a: Owner information other clinics

Navn på kanin og eier: _____

Marker det som passer / fyll inn:

- Alder: _____
- Rase: _____
- Hvor fikk du kaninen din fra?:
  - Privatperson
  - Dyrebutikk
  - Gård eller lignende
  - Dyrebeskyttelsen eller lignende
  - Annet: _____
- Bor kaninen din inne eller ute? INNE / UTE
- Bor kaninen din sammen med en annen / flere andre kaniner? JA / NEI
- Har kaninen din hatt symptomer på *E. cuniculi* tidligere? JA / NEI
  - Vanlige symptomer er blant annet: Skjevt hode, svakhet i bakparten, uvanlig mye tissing/drikking, dårlig balanse og vektnedgang.
- Har kaninen din vært behandlet for *E. cuniculi* tidligere? JA / NEI
- Har eventuelle andre kaniner i husstanden din hatt symptomer på *E. cuniculi*? (Se liste ovenfor): JA / NEI

Appendix 4b: Owner information other clinics - English

Name of rabbit and owner: _____

Mark the fitting answer / fill in the blank:

- Age: _____
- Breed: _____
- Where did you acquire your rabbit?
  - Private
  - Pet shop
  - Farm etc.
  - Animal rescue etc.
  - Other: _____
- Does the rabbit live indoors or outdoors? INDOORS / OUTDOORS
- Does the rabbit live together with other rabbits? YES / NO
- Has the rabbit ever shown symptoms of *E. cuniculi*? YES / NO
  - Common symptoms include: Head tilt, weakness in hindlimbs, more frequent urination/drinking than normal, loss of balance and weight loss.
- Has the rabbit ever been treated for *E. cuniculi*? YES / NO
- Have other rabbits in your household ever shown symptoms of *E. cuniculi*? (See list above) YES / NO

Appendix 5a: Questions regarding symptoms after measured elevated IgM

- Har kaninen vist noen symptomer på *E. cuniculi* i tiden etter prøvetaking?
  (Vanlige symptomer er typisk skjevt hode (skakker på hodet), ustøhet i bakbeina, forvirring, økt urinering eller skvetting)

- Har kaninen din blitt behandlet for *E. cuniculi* etter prøvetakingen fant sted?

- Har du andre kaniner som eventuelt har vist tegn på sykdom av *E. cuniculi*?

Appendix 5b: Questions regarding symptoms after measured elevated IgM - English

- Has the rabbit shown any symptoms of *E. cuniculi* since the date of sampling?
  (Common symptoms include: Head tilt, weakness in hindlimbs, more urination/drinking than normal, loss of balance and weight loss.)

- Has the rabbit been treated for *E. Cuniculi* since the date of sampling?

- Do you have any other rabbits that have shown signs of symptoms of *E. cuniculi*?

Appendix 6: 2x2 tables of risk factors for infection with *Encephalitozoon cuniculi* in healthy Norwegian pet rabbits with calculated P-value

Gender Male=+, female=-

	Ig+	Ig-	Total
Risk +	24	24	48
Risk -	24	14	38
Total	48	38	86
P-value	0,22		

Castrated Yes=+, No=-

	Ig+	Ig-	Total
Risk +	37	30	67
Risk -	11	8	19
Total	48	38	86
P-value	0,84		

Multi rabbits Yes=+, No=-

	Ig+	Ig-	Total
Risk +	42	32	74
Risk -	6	6	12
Total	48	38	86
P-value	0,66		

Muesli Yes=+, No=-

	Ig+	Ig-	Total
Risk +	6	2	8
Risk -	42	36	78
Total	48	38	86
P-value	0,25		

Plants Yes=+, No=-

	Ig+	Ig-	Total
Risk +	44	36	80
Risk -	4	2	6
Total	48	38	86
P-value	0,58		

Direct sun Yes=+, No=-

	Ig+	Ig-	Total
Risk +	40	35	75
Risk -	8	3	11
Total	48	38	86
P-value	0,23		

Waterbowl Yes=+, No=-

	Ig+	Ig-	Total
Risk +	38	35	73
Risk -	10	3	13
Total	48	38	86
P-value	0,96		