



UNIVERSITÀ  
DEGLI STUDI  
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Biomedicina Comparata e Alimentazione

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SCUOLA DI DOTTORATO DI RICERCA IN : SCIENZE VETERINARIE  
INDIRIZZO: SCIENZE CLINICHE VETERINARIE  
CICLO XXV

**USE OF GAMMA-GLOBULIN VALUES TO PREDICT POSITIVE IgM AND IgG SERUM FOR  
*ENCEPHALITOOZON CUNICULI* IN PET RABBITS**

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## **Abstract**

*Encephalitozoon cuniculi* (*E. cuniculi*) is a parasite that can infect a variety of animals, including immune-compromised humans. Rabbits are prone to encephalitozoonosis but the subclinical course and high serum prevalence make it difficult to diagnose with certainty in live animals. Different approaches have been used to detect the presence of an active infection in symptomatic rabbits: antibody titers, positive immunoglobulin M (IgM) and immunoglobulin G (IgG) serum for *E. cuniculi*, electrophoresis, PCR in urine, cerebrospinal fluid analysis.

In this study seventy-six rabbits showing symptoms related to this disease were divided into three groups based on their antibody results: IgM+ IgG+, IgM- IgG+, and IgM- IgG-. Statistical analysis showed that total serum protein was not effective in predicting an antibody response. In contrast the  $\gamma$ -globulin ranges from serum electrophoresis predicted whether the subject was positive for IgM and/or IgG. A significant quantitative correlation between percentage of  $\gamma$ -globulins and positive IgM and IgG serum for *E. cuniculi* was established in symptomatic rabbits. Hence, these values can be useful for screening both symptomatic and asymptomatic rabbits.

## Riassunto

*Encephalitozoon cuniculi* (*E. cuniculi*) è un parassita che può infettare diversi organismi e anche esseri umani immuno-compromessi. Sebbene i conigli siano molto suscettibili all'encefalitozoonosi, il decorso clinico, spesso asintomatico, e l'alto tasso di siero-positività rendono difficile formulare una diagnosi di certezza in vita.

Negli anni sono stati suggeriti diversi approcci per identificare la presenza di un' infezione attiva in conigli sintomatici: l'utilizzo di titoli anticorpali, la presenza nel siero di Immunoglobuline M (IgM) ed immunoglobuline G (IgG) specifiche, l'utilizzo dell'elettroforesi sierica, l'identificazione del DNA del parassita mediante PCR sulle urine e l'analisi del liquido cefalorachidiano.

In questo studio settantasei conigli che presentavano sintomi riferibili a questa malattia sono stati divisi in tre gruppi in base ai loro risultati anticorpali: IgM+ IgG+, IgM- IgG+, and IgM- IgG-. Le analisi statistiche condotte sui dati ottenuti hanno evidenziato che le proteine sieriche totali non sono utili nel predire la risposta anticorpale. Di contro i valori delle  $\gamma$ -globuline ottenuti mediante l'elettroforesi sierica sono stati in grado di predire la presenza di IgM e/o IgG. È stata pertanto trovata una correlazione significativa e quantitativa tra le percentuali delle  $\gamma$ -globuline e la presenza o meno di IgM e IgG per *E. cuniculi* nel siero di conigli sintomatici.

Questi risultati possono essere utili per eseguire uno screening sia nei soggetti sintomatici sia che in quelli asintomatici.

## **Structured summary**

*Objectives:* To establish whether there is a significant quantitative correlation between changes in serum proteins and positive IgM and/or IgG serum for *E. cuniculi* in rabbits showing symptoms related to encephalitozoonosis. In particular, total serum protein levels and the percentage of  $\gamma$ -globulins in the electrophoresis pattern were statistically compared to antibody results.

*Methods:* Seventy-six rabbits showing symptoms related to the disease were tested for IgM and IgG antibodies and total protein, and serum electrophoresis was performed. Three groups of rabbits were established based on antibody results: IgM+ IgG+, IgM- IgG+ and IgM- IgG-. A one-way ANOVA was used to statistically compare the data using the total serum protein and the percentage of  $\gamma$ -globulins in the electrophoretic pattern as the only variables.

*Results:* Total serum protein is not effective in predicting an antibody response to *E. cuniculi*, and pet rabbits showing symptoms related to encephalitozoonosis that were positive or negative for IgM and/or IgG did not show significant changes in their total serum protein values. In contrast, the  $\gamma$ -globulin ranges from serum electrophoresis can predict whether the patient is positive for IgM and/or IgG.

*Clinical Significance:* These diagnostics may serve as useful screening tests both for symptomatic and asymptomatic rabbits that will aid in the early diagnosis of the disease

## **Introduction**

### State of the art of the disease

*Encephalitozoon cuniculi* (*E. cuniculi*) is an obligate intracellular microsporidian parasite that can infect a variety of animals, including immune-compromised humans (Jeklova *et al.* 2010a). Considering the potential zoonotic risk of *E. cuniculi*, serological screening is important for both symptomatic and asymptomatic animals.

Three different strains of *Encephalitozoon cuniculi* have been isolated up to now (Didier *et al.* 1995). Strain 1 has been associated with rabbits, strain 2 with rodents, and strain 3 with dogs. Differences are only in their DNA and it is still not known if they are species specific or if they can transfer to different hosts. Interestingly, in humans strain 3 has been isolated in the USA, and strain 1 in Europe (Valencakova 2008)

### Spread of the infection in rabbits and biological cycle of the parasite

Rabbits are usually infected by ingesting and/or inhaling spores from infected rabbit urine.

The spore possesses a polar filament that extrudes into host intestinal mucosal cells, injecting spore contents and initiating infection. Multiplication of the *E. cuniculi* organism takes place in host alimentary cell vacuoles, with eventual cell rupture and spore invasion of the reticulum-endothelial system and systemic circulation by infected macrophages. Further organism multiplication occurs via ordinary fission or schizogony within vacuoles or pseudo cysts (schizonts) found in reticulum-endothelial cells of target organs. Spores eventually develop and, with time, the pseudo cyst becomes overcrowded and ruptures.

Vertical transmission has also been confirmed by demonstrating the presence of *E. cuniculi* DNA in the placentas and fetuses of seropositive dams (Baneux and Pognan 2003). Under experimental

conditions, intravenous, intra-peritoneal, intra-tracheal, intra-cerebral and ocular infections have also been reported (Jeklova *et al.* 2010a).

### Clinical course of the infection and associated clinical signs

Although the disease may be the result of inflammation caused by the organism, it is not clear whether the causative organism is present when symptoms appear. Cell rupture caused by the parasite is associated with a chronic inflammatory response, and most immunocompetent rabbits develop subclinical infections in a balanced host-parasite relationship. These infections are associated with granulomatous lesions that primarily affect the brain, kidney or eyes (Fisher and Carpenter 2012). In rabbits, the course of the disease is usually chronic, but in some cases, the onset of clinical signs is sudden and often occurs after a stressful situation.

Target organs for the organism include eye lens, central nervous system and kidneys; associated lesions are granulomas meningoencephalitis, chronic interstitial nephritis and phacoclastic uveitis (Künzel and Joachim 2010).

In a study of 191 pet rabbits with suspected encephalitozoonosis at the University of Vienna (Austria), the animals that tested positive for antibodies against *Encephalitozoon cuniculi* showed mainly neurological symptoms and in particular vestibular disease. Phacoclastic uveitis and renal failure were diagnosed in a lower percentage in this study (Künzel et al 2008). Other prior studies in different countries showed similar results.

In the case of vestibular disease, depending on the severity of the head tilt (Fig. 1), circular movements, falling, or rolling can be observed. Other common neurological symptoms that can be observed are: ataxia with or without urinary incontinence, paresis or paralysis, nystagmus, and seizures.

Dermatitis of the perineum can be observed in the case of incontinence due to neurological involvement and in the case of polyuria due to renal disease, (Fig. 2) (Fisher and Carpenter 2012).

Phacoclastic uveitis is the consequence of intrauterine infection and is characterized by the infiltration of the eye lens by various inflammatory cells (granulocytes, macrophages, giant cells) leading to a rupture of the lens capsule (Fig. 3, 4). Iris and ciliary body are infiltrated by plasma cells and lymphocytes. Parasites are only found in the lens (Giordano et al. 2005).

In addition to uveitis, cataracts of various degrees can also be diagnosed with ophthalmological examination (Künzel and Joachim 2010).





Fig. 1: pet rabbit showing moderate head tilt



Fig. 2: pet rabbit with obvious signs of perineal dermatitis caused by poliuria and incontinence



Fig. 3: initial stage of phacoclastic uveitis in a pet rabbit



Fig. 4: advanced stage of phacoclastic uveitis in a pet rabbit

### Differential diagnosis

The definitive diagnosis of encephalitozoonosis *in vivo* is difficult because related symptoms are seen in other diseases too. Radiography of the skull can help rule out an otitis media, mainly caused by *Pastourella multocida*, that represents the main differential diagnosis in the case of vestibular disease. Computer Tomography and Magnetic Resonance Imaging allow diagnosis of intracranial masses that could explain other neurological signs.

Moreover toxicosis (e.g. fipronil), trauma, heat stroke, degenerative and nutritional diseases display similar neurological symptoms interfering with the diagnosis.

*Toxoplasma gondii*, cerebral larva migrans (*Baylisascaris sp.*) and larvae of *Cutebra* species are included in the list of differential diagnoses, as well as *Herpes simplex* encephalitis, which has been associated with close contact with humans with *herpes labialis* (Fisher and Carpenter 2012).

*Phacoclastic uveitis* caused by *Encephalitozoon cuniculi* in rabbits is seen mainly in young rabbits and in contrast to other animals the capsule rupture is not induced by a traumatic insult. Other possible causes can be: bacteria (e.g.. *Pastourella multocida*), keratits, trauma and/or foreign bodies.

### Electrophoresis

Protein electrophoresis has been previously described as a sensitive assay for the inflammatory and infectious process in mammals, and for this reason it has been investigated to see if it could help in the diagnosis of encephalitozoonosis in live rabbits.

The serum protein profiles are mostly defined using routine serum protein electrophoresis, which allows identification of different fractions or regions, each being composed of one or several proteins, with similar electrophoresis mobility. Different macromolecules in the mixture will

migrate at different speeds, depending on the nature of the gel and the physical-chemical characteristics of the macromolecules.

Four fractions can be identified: albumin,  $\alpha$ ,  $\beta$  and  $\gamma$ -globulins. Both  $\alpha$  and  $\beta$  can be divided respectively into  $\alpha_1$  and  $\alpha_2$  and in  $\beta_1$  and  $\beta_2$ -globulins.

Albumin is the main fraction of the plasma proteins and represents the main and more homogeneous peak in the electrophoretograms of all species. The main functions of albumin are to carry many molecules and maintain the oncotic pressure of the blood. The  $\alpha$ ,  $\beta$  and  $\gamma$ -globulins are a smaller fraction responsible for the humoral response of the organism. While  $\alpha$  and  $\beta$  are characteristic of the acute phase of the flogosis, the  $\gamma$  are present in the chronic phase. To identify and separate these fractions in the electrophoresis pattern some rules have to be followed:

- ✓ the fractions always appear in the same order: albumin,  $\alpha$ ,  $\beta$  and  $\gamma$ -globulins,
- ✓ albumin is identified as the peak nearest the anode, and usually appears as a high and narrow peak,
- ✓ the midpoint on the horizontal axis of the electrophoretogram is the approximate separation between  $\alpha$  and  $\beta$ -globulins,
- ✓ the separation between  $\alpha_1$  and  $\alpha_2$ -globulins coincides with the major valley in the  $\alpha$ -globulins fractions,
- ✓ the separation between  $\beta$  and  $\gamma$ -globulins is established as the most marked valley that appears between the  $\beta$  and  $\gamma$ -globulins,
- ✓ to determine the end of the  $\beta$ -globulin area it is useful to know that the  $\beta$ -globulin area is of similar size to  $\alpha$ -globulin area,
- ✓ sub-fractions in  $\beta$  and  $\gamma$ -globulins can be established if valleys appear inside these fractions (Ceròn et al 2010).

### Different types of electrophoresis

Different types of electrophoresis exist on the market and each one presents some advantages and disadvantages. Although Cellulose Acetate Electrophoresis is inexpensive and allows a fast electrophoresis separation, several time-consuming procedures are required and high-resolution separation is impossible

Agarose gel Electrophoresis is the one most commonly used in veterinary medicine. The procedure is labour-intensive but the introduction of prepackaged gels and integration of computers make it easier. It has much better resolution that allows the detection of several protein bands and the medium is clear after drying, which allows very sensitive densitometry measurements. This type is expensive and the medium is fragile so it must be handled carefully.

Polyacrylamide gel separates more serum protein fractions and supporting substances, is less fragile than agarose gel, and it is transparent, which allows densitometry reading. However, this technique is relatively labor intensive for routine applications.

Capillary electrophoresis allows high-resolution separation of both inorganic and organic ions including nucleic acids, proteins and peptides. This new technique is faster, needs a small amount of sample, and can be completely automatic. In this system, the classic technique of electrophoresis is carried out in a small capillary tube that serves as the electrophoresis chamber, connected to a detector, and via buffer reservoirs to a high voltage power supply (Ceròn et al 2010).

### Goals in the in vivo diagnosis of *Encephalitozoon cuniculi*

The subclinical course and high serum prevalence in pet rabbits make it difficult to diagnose *E. cuniculi* with certainty in live animals. High titers in the *E. cuniculi* ELISA (>1:1024) and specific changes in serum electrophoresis (a decreased Albumin/Globulin ratio, decreased  $\alpha$ 2-globulins and increased  $\gamma$ -globulins) may support the presence of an active infection. Therefore, the combined use of the *E. cuniculi* ELISA test and serum electrophoresis may help in the antemortem diagnosis of

infection in rabbits (Cray 2009). However, antibody titers only indicate exposure and the response to exposure. They do not reveal infection. A rabbit could have been exposed to the organism and produced a successful immune response, thus preventing the development of an infection. That rabbit would have a positive titer yet was never infected. In contrast, a rabbit may have been infected, but the immune response did not increase antibody titers to a level sufficient for detection because of concurrent immune depression in the animal (Rosenthal 2007). For this reason, the simultaneous testing of IgM- and IgG-specific antibodies may indicate a patient's infection status. The presence of IgM may indicate an acute infection, while the presence of only IgG may denote a chronic or latent infection. The presence of both IgM and IgG may indicate an acute infection, a reactivated infection or a re-infection (Jeklova *et al.* 2010b).

In the case of a positive antibody reaction in rabbits showing associated clinical signs, it is still impossible to definitively diagnose *E. cuniculi* as the causative agent because a high serum prevalence exists in clinically healthy rabbits, with high titers fluctuating for several years even in the absence of clinical signs (Jeklova *et al.* 2010b). A negative test result is generally the most useful serological test result because it usually rules out encephalitozoonosis, but only in chronic cases rather than in acute ones (Harcourt-Brown 2010).

In general, maternal antibodies against *E. cuniculi* are passed on to the offspring and are present until 4 weeks of age in rabbits (Lyngset 1980). For this reason, it is not appropriate to test a 4-week-old or younger rabbit born to a seropositive dam. In the case of trans-placental transmission, the parasite can be recognised by the foetal immune system as “self,” making the diagnosis even more difficult *in vivo* because both serological and electrophoretic screening are not effective. In these cases, only PCR can identify *E. cuniculi* spores in urine, although the shedding of the spores is sporadic. A positive test result indicates only that the rabbit is shedding the parasite and represents a source for the infection of other animals, but this result does not confirm a clinical disease.

Cerebrospinal fluid analysis in rabbits with vestibular disease and/or paresis due to clinically suspected encephalitozoonosis was characterised by lymphomonocytic pleocytosis (Jass *et al.* 2008). In the opinion of the authors, this diagnostic technique carries high risk due to its surgical and anaesthetic procedures, and these cytological changes can be observed with other diseases too. For these reasons, it is not considered a useful diagnostic for pet rabbits.

**Aim of the study**

The aims of this study were to establish whether there is a significant quantitative correlation between changes in serum protein levels and the percentage of  $\gamma$ -globulins in the serum electrophoretic pattern and to determine whether IgM and/or IgG antibodies are present in rabbits showing specific symptoms related to encephalitozoonosis. This information will help develop a diagnostic plan with reference ranges useful for a complete *E. cuniculi* screening in pet rabbits.



## Material and Method

From 01/01/2012 to 01/01/2013, 76 rabbits showing neurological, renal and ocular signs likely related to *E. cuniculi* were examined. The animals were aged between 2 months and 8 years old and were intact or neutered males and females. Their owners first noticed the clinical signs developing less than one week prior to the veterinary consult. During each examination, a blood sample was obtained from the saphenous vein. The sample was centrifuged to obtain serum, which was refrigerated and sent within 24 hours to a veterinary analysis laboratory to perform electrophoresis in an agarose gel (Hydrasys 2 machine, Hydragel reagents and Phoresis software, Sebia Italia srl. Bagno a Ripoli, Florence, Italy) and to test for IgG and IgM antibodies against *E. cuniculi* using the indirect fluorescent-antibody (IFA) method (Fluo Encephalitozoon cuniculi and coniugato rabbit IgM, Agrolabo, Scarmagno, Turin, Italy). Total serum proteins were also quantified using liquid chemistry (Hitachi Boheringer Mannheim, SD Servizi Diagnostici, Roma, Italy). All haemolytic or lipemic serum samples were not used for these analyses. Haemolysis and lipemia produced a change in electrophoretogram morphology in canine samples analysed by capillary zone electrophoresis (CZE), giving an interference peak located in the  $\beta$  region when haemoglobin was increased and in the  $\alpha_1$  region when lipids were increased. Bilirubin produced an increase in albumin and  $\alpha_1$  and a decrease in the  $\alpha_2$  and  $\beta_2$  fractions. Fibrinogen did not produce any additional peaks during CZE, so no differences were noted between the serum and plasma samples (Martinez-Subiela *et al.* 2002).

Animals with extreme values (lower or higher values of  $\gamma$ -globulins) were removed from the statistical analysis, and the remaining animals were divided into three different groups based on their serological results: 26 samples were positive for IgG and IgM (IgM+ IgG+), 25 samples were negative for IgM and positive for IgG (IgG+) and 19 samples were negative for both immunoglobulins (IgM- IgG-).

The one-way ANOVA was used to statistically compare data considering each of two variables individually: the total protein expressed in g/dl (TP) and the percentage of  $\gamma$ -globulin in the serum electrophoretic pattern (%  $\gamma$ -globulins). The Statview 5.1 software was used to calculate the mean table, ANOVA table, Fisher PLSD test and interaction graphs for both variables, as shown below.

## Results

### Statistical analysis of serum total protein

Considering total serum proteins as the only variable the following results were obtained: Table. 1, 2,3 and Graph 1

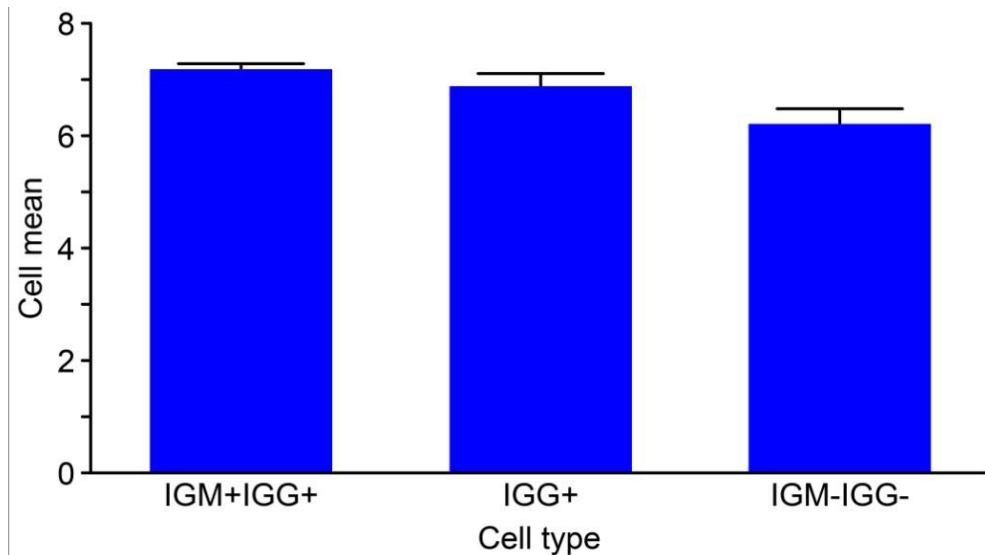
Groups	Count	Mean	Std. Dev.	Std. Err.
IgM+ IgG+	26	7,16	0,762	0,149
IgG+	25	6,892	0,958	0,192
IgM- IgG-	19	6,189	1,235	0,283

	DF	Sum of square	Mean square	F-value	P-value	Lambda	Power
Groups	2	10,620	5,310	5,559	0,0058	11,118	0,850
Residual	67	64,000	0,955				

Groups	Mean Diff.	Crit. Diff.	P-Value	
IgM+ IgG+, IgG+	0,266	0,546	0,3353	Not Significant
IgM+ IgG+, IgM- IgG-	0,968	0,586	0,0016	Significant
IgG+, IgM- IgG-	0,703	0,594	0,0211	Significant

Graph 1: Interaction graphic for serum total protein

Error bars:  $\pm$  Std. Err.



The following results were obtained by comparing the total serum protein among the three groups. The “IgM+ IgG+” group compared to the “ IgM- IgG-” group was significantly different ( $p < 0.05$ ). The “IgM+ IgG+” group compared to the “IgG+” group was not significantly different ( $p = 0.21$ ). The “IgG+” group compared to the “IgM- IgG-” group was significantly different ( $p < 0.05$ ).

The ranges of total protein values for all three groups did not significantly exceed the normal physiological range of 5.4 g/dl to 7.5 g/dl (Carpenter 2013).

The “IgM+ IgG+” group had a range of  $7.16 \pm 0.762$  g/dl.

The “IgG+” group had a range of  $6.89 \pm 0.958$  g/dl.

The “IgM- IgG-” group had a range of  $6.19 \pm 1.235$  g/dl.

### Statistical analysis of serum $\gamma$ -globulins

Considering the percentage of  $\gamma$ -globulins in the serum electrophoresis tract as the only variable the following results were obtained: Table 4,5,6 and Graph. 2

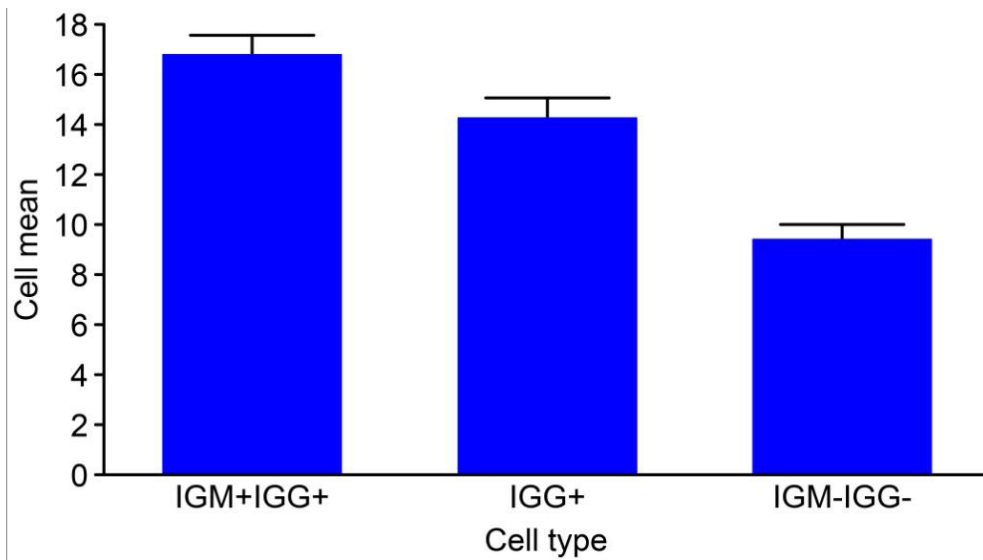
Groups	Count	Mean	Std. Dev.	Std. Err.
IgM+ IgG+	26	16,792	3,810	0,744
IgG+	25	14,216	4,288	0,858
IgM- IgG-	19	9,442	2,538	0,582

	DF	Sum of square	Mean square	F-value	P-value	Lambda	Power
Groups	2	597,545	298,773	21,753	<0,0001	43,507	1,000
Residual	67	920,218	13,753				

Groups	Mean Diff.	Crit. Diff.	P-Value	
IgM+ IgG+, IgG+	2,576	2,072	0,0156	Significant
IgM+ IgG+, IgM- IgG-	7,350	2,233	<0,0001	Significant
IgG+, IgM- IgG-	4,774	2,251	<0,0001	Significant

Graph 2: Interaction graphic for  $\gamma$ -globulins

Error bars:  $\pm$  Std. Err.



The following results were obtained when comparing the  $\gamma$ -globulin percentages from serum electrophoresis among the same three groups.

The “IgM+ IgG+” group compared to the “IgM- IgG-” group was significantly different ( $p < 0.001$ ).

The “IgM+ IgG+” group compared to the “IgG+” group was significantly different ( $p < 0.0156$ ).

The “IgG+” group compared to the “IgM- IgG-” group was significantly different ( $p < 0.001$ ).

The normal physiological percentage for  $\gamma$ -globulins ranges between 8.6% and 9.6% (Melillo 2013).

The “IgM+ IgG+” group had a  $\gamma$ -globulin range of  $16.79 \pm 3.81\%$ .

The “IgG+” group had a  $\gamma$ -globulin range of  $14.22 \pm 4.3\%$ .

The “IgM- IgG-” group had a  $\gamma$ -globulin range of  $9.44 \pm 2.54\%$ .

### **Statistical acronyms**

- ✓ DF (Degree of Freedom) is the number of values in the final calculation of a statistic that are free to vary.
- ✓ Sum of squares is a statistical technique used in regression analysis to determine the dispersion of data points.
- ✓ Mean square is an estimation of the population variance based on the variability.
- ✓ F-value is used to test the significance of adding new model terms to those terms.
- ✓ P-value is the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true.
- ✓ Lambda is a test statistic used in multivariate analysis of variance to test whether there are differences between the means of identified groups of subjects on a combination of dependent variables.
- ✓ Power is a number that indicates a study will obtain a statistically significant effect.
- ✓ Std. Dev. (Standard Deviation) shows how much variation or dispersion exists from the average (mean), or expected value. A low standard deviation indicates that the data points tend to be very close to the mean; high standard deviation indicates that the data points are spread out over a large range of values.
- ✓ Std. Err. (Standard Error) is the standard deviation of the sampling distribution of a statistic.
- ✓ Fisher's PLSD Test is used to determine if there is a non-random association between two categorical variables
- ✓ Crit. Diff. (Critical Difference) is required to call a pair of means or totals significantly different, and is what differentiates all pair-wise tests from each other. If the difference obtained is greater than or equal to the critical difference, it is significant, otherwise there is a non-significant difference between the pairs of scores.

## Discussion

Total serum protein is not effective in predicting an antibody response to *E. cuniculi*, and pet rabbits showing symptoms related to encephalitozoonosis that were positive or negative for IgM and/or IgG did not show significant changes in their total serum protein values relative to the reference ranges.

In contrast, the  $\gamma$ -globulins ranges from serum electrophoresis can predict whether the patient is positive for IgG and/or IgM immunoglobulins.

In particular:

$\gamma$ -globulins of  $16.79 \pm 3.81\%$  indicate *E. cuniculi* IgG and IgM positive rabbits

$\gamma$ -globulins of  $14.22 \pm 4.3\%$  indicate *E. cuniculi* IgG positive rabbits

$\gamma$ -globulins of  $9.44 \pm 2.54\%$  indicate chronically diseased rabbits with negative *E. cuniculi* IgG and IgM.



## **Conclusion**

Although an increase in total serum protein is not observed during the early diagnosis of encephalitozoonosis in rabbits, indicators such as the  $\gamma$ -globulins increase, as already reported in other work. In symptomatic animals, the percentage of  $\gamma$ -globulins in the electrophoretogram increases when the IgM and IgG are positive; thus, the electrophoretogram has a predictive value for the serological response. For this reason, serum electrophoresis can be used as reliable diagnostic information, even if it is not highly specific.

## **Acknowledgements**

The authors thank Prof. Marco Martini, Alessandro Zanella and Alessia Cristofoli for statistical analysis, Dr. Paolo Romano and LAGVET veterinary laboratory for performing all laboratory analysis and Dr. Irene Vazzana (IZS Sicilia) for technical support.

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