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ORIGINAL PAPER

Application of Western blot analysis for the diagnosis of *Encephalitozoon cuniculi* infection in rabbits: example of a quantitative approach.

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Abstract Diagnosis of *Encephalitozoon cuniculi* infection in rabbits remains a major veterinary issue. ELISA or immunofluorescence assays are the current reference standards of serological tests. However, these conventional techniques suffer from a lack of accuracy for distinguishing active from past infections, as a positive serostatus is common in clinically normal rabbits. In this study, we assessed the diagnostic performance of Western blot (WB) to detect both anti-*E. cuniculi* immunoglobulin G (IgG) and immunoglobulin M (IgM) in comparison with ELISA and to address the intensity of the immune response through a quantitative approach. Positive WB results were highly correlated with the *E. cuniculi*-related diseased status ($P < 0.0001$). Although it was more labor intensive and less standardized, quantitative WB provided detailed comparable analysis regarding the humoral response and diagnostic performance similar to ELISA testing with statistically higher sensitivity (88.4 vs. 76.1% for IgG detection and 84.3 vs. 70.4% for IgM, $P < 0.01$). Several specific WB bands were shown to be significantly associated with concomitant clinical signs, like the one located at 50 kDa (OR = 8.2, [2.4–27.7], $P = 0.0008$) for IgG and (OR = 27.9, [4.2–187.9], $P = 0.0006$) for IgM. Therefore, the quantitative WB may have application in veterinary diagnostic laboratories to increase the accuracy of the clinical diagnosis of *E. cuniculi* infection. In addition, this tool may help to further understand the development and function of the humoral immune response to this infectious agent.

Keywords *Encephalitozoon cuniculi* · Microsporidiosis · Rabbits · Diagnosis · Western blot · Elisa · CRP

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Introduction

Encephalitozoon cuniculi is a unicellular microorganism related to fungi that invades mammalian cells where its spores thrive (Mathis et al. 2005; Didier and Weiss 2006). *E. cuniculi* infection is a great concern in pet rabbits in which seroprevalence rates have been estimated from 37 to 68% (Harcourt-Brown and Holloway 2003; Cray et al. 2009; Künzel and Joachim 2010; Keeble 2011; Cray et al. 2015). It may be either subclinical or clinical with acute signs or chronic expressions which define the active disease called encephalitozoonosis (Keeble 2011). Kidneys are considered a primary target tissue, and neurological signs due to that granulomatous encephalitis are subsequently reported during later stages (Künzel and Joachim 2010). However, except for phacoclastic uveitis, no clinical sign is considered pathognomonic enough to exclude differential diagnoses including bacterial infection or injury. Definitive *antemortem* diagnosis is difficult because *E. cuniculi* spores shedding in excreta are intermittent and their detection is therefore unreliable (Csokai et al. 2009; Künzel and Joachim 2010). Current serology techniques include enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody test

(IFAT) (Boot et al. 2000). In studies of experimental and natural infections, serology tests were shown to correlate well with histopathological lesions (Waller 1977; Cox et al. 1979; Csokai et al. 2009). However, there is difficulty in distinguishing between recent or past *E. cuniculi* infection since antibodies remain for several years, including immunoglobulin M (IgM) that are usually detectable up to 18 weeks post-exposure (Khan et al. 2001; Latney et al. 2014). Recently, C-reactive protein (CRP) measurement has been proposed in association with the ELISA tests to increase their specificity for detecting current infection (Cray et al. 2015). Except for diagnosis of phacoclastic uveitis, polymerase chain reaction (PCR) has not been routinely implemented, and preliminary evidence suggests a low sensitivity (Csokai et al. 2009). Consequently, specific diagnosis can be difficult to obtain and therefore fatal outcomes and advanced disease are frequent: in some studies, it has been reported that rabbits overall recover in only 50% cases when undergoing anti-*E cuniculi* treatment, although the results actually depend on the affected organs, since uveitis and encephalitis may be considered globally more curable than chronic renal failure (Künzel et al. 2008).

Thus, new tools are needed to enhance the diagnostic accuracy of *E. cuniculi* infection in rabbits but also in other species (Künzel et al. 2014). The goal of this study was to assess the feasibility and the diagnostic performance of Western blot (WB) analysis to detect both specific anti-*E. cuniculi* immunoglobulin G (IgG) and IgM antibodies and to quantify the level of immune response in rabbits. Results were compared to a previously validated ELISA method of IgG and IgM quantitation.

Material and methods

Study population: animals and samples

Rabbit samples were obtained from a national multicenter study which systematically included patients with suspected encephalitozoonosis from 46 veterinary clinics in the USA over two periods, February 2015 to August 2015 and February 2016 to August 2016, at the time of their first medical visit. The probable diagnosis of presumptive encephalitozoonosis was made, or rejected, upon results of standard clinical examination and a questionnaire completed by the submitting practitioners independent of the laboratory tests of this study. This survey recorded descriptions of the clinical signs upon presentation, other diagnostic evaluations like CRP measurement in blood, and response to treatment suitable for *E. cuniculi* infection when undertaken i.e., benzimidazoles.

Adjustment with control subjects was initially made by matching on age ± 1 year and sex, in a ratio 1:1. The controls were either clinically normal or currently ill for reasons other than active encephalitozoonosis, whatever their medical history and serostatus.

All submissions used in this study were part of routine or specific diagnostic sampling sent to the Division of Comparative Pathology at the University of Miami for analysis.

Serological tests

ELISA

Briefly, 50 μL of serum, tested at a 1:64 screening dilution in milk 5%-phosphate-buffered saline-Tween 0.05%, were placed in duplicate in wells of *E. cuniculi* antigen-coated 96-well microtiter plates (Charles River, San Diego CA, USA). After incubation at 37 °C for 30 min, 50 μL of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:4000, Sigma-Aldrich, St. Louis, MO, USA) was added into the first well, and 50 μL of anti-rabbit IgM (1:4000, Bethyl, Montgomery, TX, USA) was put into the second one. Following another 30-min incubation and a washing step, 50 μL of chromogen ABTS® solution (KPL, Gaithersburg MA, USA) was added to each well. The optical density related to reaction intensity after 10 min was read at 405 nm in a SpectraMax® microplate spectrofluorometer (Molecular Devices, Downingtown, PA). A net score of 3 or above for IgG and of 2.5 or above for IgM was considered positive. Positive samples were diluted 1:64 to 1:8192 to determine the titers. Positive cutoff values were set at 1:512 and 1:64 for IgG and IgM, respectively (Cray et al. 2015). Results were interpreted in association with CRP concentration that had been also measured in every rabbit blood on Daytona® instrument (Randox, Kearneysville, WV, USA), in the same lab (Cray et al. 2015).

Western blotting

The WB analysis was carried out as previously described (Künzel et al. 2014). Briefly, supernatants were collected from the *E. cuniculi* strain CH-K 2169 cultured in Madin-Darby Canine Kidney (MDCK) cells (ATCC® Number CCL-34) (Künzel et al. 2014). After a washing step in Hank's Balanced Salt Solution (HBSS, PAA, Pasching, Austria), followed by a treatment with 0.25% sodium dodecyl sulfate (SDS) to dissolve the MDCK cells and three washing steps with HBSS, *E. cuniculi* spores were disrupted in a reducing buffer containing 0.0625 M Tris-HCl and 2% SDS by homogenization, followed by thawing-freezing cycles in liquid nitrogen, then centrifugation. The protein fraction was re-suspended in 100 mM dithiothreitol (DTT) and 2.5% SDS and separated on a 12% polyacrylamide gel in SDS-reducing buffer containing 5% ß-mercaptoethanol at 200 V/200 mA for 1 h and

Table 1 Overall characteristics of the included rabbit patients in the final cohort

	Mean (± Standard deviation) or number (%) [95%confidence interval]	
Study population (N = 153)	*E. cuniculi* diseased cases (N = 72)	Non-*E. cuniculi* diseased controls (N = 81)
Age (years)	5.8 years (±2.9) [5.2–6.5 years]	4.3 years (±3.7) [3.5–5.2 years]
Gender (male sex)	43 (59.7%) [48.4–71.1%]	46 (56.8%) [46.0–67.6%]
Clinical course including the following symptoms[a]:	61 (84.7%) [76.4–93.0%]	55 (67.9%) [57.7–78.1%]
Neurological[b]	36 (55.4%) [43.3–67.5%]	35 (55.6%) [43.3–67.8%]
Digestive[c]	16 (24.6%) [14.1–35.1%]	15 (23.8%) [13.3–34.3%]
Urinary[d]	14 (21.5%) [11.5–31.5%]	3 (4.8%) [0.0–10.0%]
Ocular[e]	11 (16.9%) [11.5–31.5%]	5 (7.9%) [1.3–14.6%]
Other[f]	9 (13.8%) [5.5–22.2%]	6 (9.5%) [2.2–16.8%]
Biological findings		
IgG ELISA titer (1:dilution)	1024 (1792) [0–8192][g]	0 (0) [0–2048][g]
IgM ELISA titer (1:dilution)	128 (96) [0–512][g]	0 (0) [0–256][g]
C-reactive protein (ng/mL)	39.3 (±54.2) [26.6–52.0]	7.1 (±29.2) [0.6–13.5]
Anti-*E. cuniculi* curative therapy including the following drug(s)[a]:	53 (73.6%) [63.4–83.8%][h]	11 (13.6%) [6.1–21.0%]
Oxibendazole	9 (14.5%) [5.7–23.2%]	8 (9.9%) [3.4–16.4%]
Fenbendazole	29 (46.8%) [34.4–59.2%]	3 (3.7%) [0.0–7.8%]
Albendazole	2 (3.2%) [0.0–7.6%]	/
Other	13 (21.0%) [10.8–31.1%]	1 (1.2%) [0.0–3.6%]
Total duration of anti-*E. cuniculi* treatment (days)	83.1 days (±137.9) [43.1–123.2 days]	6.3 days (±6.0) [2.6–10.3 days]

Because of non-completion of the approval form, ten subjects had to be excluded from the *E. cuniculi*-diseased group and one from the control group after their initial enrollment. The group assignment was made according to the clinical diagnosis, including C-reactive protein measurement with equivocal case (for details, see "Methods" section). Based on clinical signs, 11 non-*E. cuniculi* diseased controls received preliminary short-term *E. cuniculi*-targeted treatment until a definitive alternative diagnosis was reached

ELISA enzyme-linked immunosorbent assay, *N* number, *SD* standard deviation, / 0 (0.0%) [0.0–0.0%]

[a] Including not only "combined treatment", but also "combined clinical signs"

[b] That is, head tilt, ataxia, circling, nystagmus, rotational/rolling/swaying movements, torticollis, seizure, cortical blindness, abnormal spine reflexes, paresis, head tremors/nodding

[c] That is, chronic/recurring gastrointestinal disease (e.g., stasis), weight loss, etc.

[d] That is, polyuria, polydipsia, dehydration, osteodystrophy

[e] That is, unilateral phacoclastic uveitis, cataracts, endophthalmitis

[f] That is, lethargy, aggression, auto-mutilation, excessive running/jumping, etc.

[g] Median (interquartile range) [min.–max. value]

[h] 86.8% which received specific anti-*E. cuniculi* therapy improved their health status; four patients were lost to follow-up

30 min (Künzel et al. 2014). *E. cuniculi* proteins were electrophoretically transferred to pure nitrocellulose membranes (pore size 0.45 μm; Bio-Rad, Hercules CA, USA) using a Mini-PROTEAN 3® Cell blotting system (Bio-Rad, Hercules CA, USA) at 35 V and 150 mA, for 3 h. After blocking non-specific binding sites and a subsequent incubation for 1 h with the test samples in a dilution of 1:300, the membrane was cut into strips and treated with either HRP-conjugated goat anti-rabbit IgG (diluted 1:650, Sigma-Aldrich, St. Louis, MO, USA) or HRP-conjugated goat anti-rabbit IgM (1:1200, Bethyl, Montgomery, TX, USA) for 1 h at room temperature. Immunodetection was finally achieved when the WB bands were detected with a DAB-peroxidase substrate kit (KPL, Gaithersburg MA, USA).

Interpretation and data analysis

For WB, each sample was examined in triplicate and independently read by two experts. For every run, a highly *E. cuniculi*-positive control serum was used as reference. According to our preliminary assays and previously published works, a specific focus was made on seven WB bands located at 135, 75, 50, 40, 30, 25, and 19–20 kDa (Künzel et al. 2014). By adding the intensity results of each abovementioned band, which was individually scored from 0 to 4, the WB global intensity for every sample was thus ranked on 28 points (Oliva et al. 2015).

Statistical analyses were performed using XLSTAT v.2016.6.04® software (Addinsoft, Paris, France). Receiver operating characteristic (ROC) curves for IgG and IgM, and their

respective areas under the curve (AUCs), were generated in order to establish positive cutoffs with the WB score. Lower and upper cutoff values were calculated using Youden's J statistic. A true positive was defined as a positive result that was in agreement with the definitive diagnosis of active encephalitozoonosis according to the clinical examination associated with those from the clinical questionnaire and CRP measurement. Diagnostic measures were then calculated to assess performance: sensitivity (Se), specificity (Sp), positive and negative predictive values (PPV and NPV), and likelihood ratios (LR+ and LR−). The comparison of diagnostic performance with the routine ELISA kit was made using the McNemar and Wilcoxon signed-rank tests. The Pearson's rank correlation coefficient was used to assess the statistical associations. Statistical association was tested by regression models, expressed in odds ratio (OR) with 95% confidence interval ($_{95\%}$CI). Missing data, e.g., when the total volume of the sample was insufficient to complete all the analyses, were managed by the method of mean imputation. The α-risk was adjusted at 0.05.

Results

Study population: animal and samples

In total, 153 individual samples were examined. The demographics of the included patients are summarized in Table 1. In the *E. cuniculi* diseased group, 72 cases were recruited on the basis that they were probable cases of encephalitozoonosis. Among those which were treated with anti-*E. cuniculi* drugs, 86.8% recovered. In the non-*E. cuniculi* diseased group, 81 animals served as controls.

Interpretation of the quantitative Western blot testing

Overall, WB of positive *E. cuniculi* samples confirmed that proteins of approximately 135, 75, 50, 40, 30, 25, and 19–20 kDa were consistently observed in seropositive animals (Fig. 1): for IgG antibody detection, presence of the bands at 135 and 50 kDa had the highest sensitivity (estimated at 91.4% for both), while all bands showed specificities above 95%. The sensitivities were lower for IgM detection: the highest was 77.1% for the 50-kDa band. However, each band had high specificities, ranging from 96.3%, for the 26-kDa band, to 100.0%, for the 80-, 38-, and 35-kDa bands.

Presence of IgM directed against the 135-kDa band (OR = 4.0, [1.3–12.3], P = 0.02), 75-kDa band (OR = 8.6, [2.7–27.4], P = 0.0003), 50-kDa band (OR = 8.2, [2.4–27.7], P = 0.0008), 40-kDa band (OR = 5.8, [2.0–16.9], P = 0.001), and 25-kDa band (OR = 5.5, [1.9–15.9], P = 0.002), as well as the IgG anti-50-kDa band (OR = 27.9, [4.2–187.9], P = 0.0006) and anti-30-kDa band (OR = 5.0, [1.3–19.5], P = 0.02), was found to be statistically associated with the report of current clinical signs in the diseased group. However, during encephalitozoonosis, no one WB band was particularly more associated with renal signs in comparison with neurological symptoms and, conversely, no band was able to specifically differentiate neurological encephalitozoonosis versus renal disease.

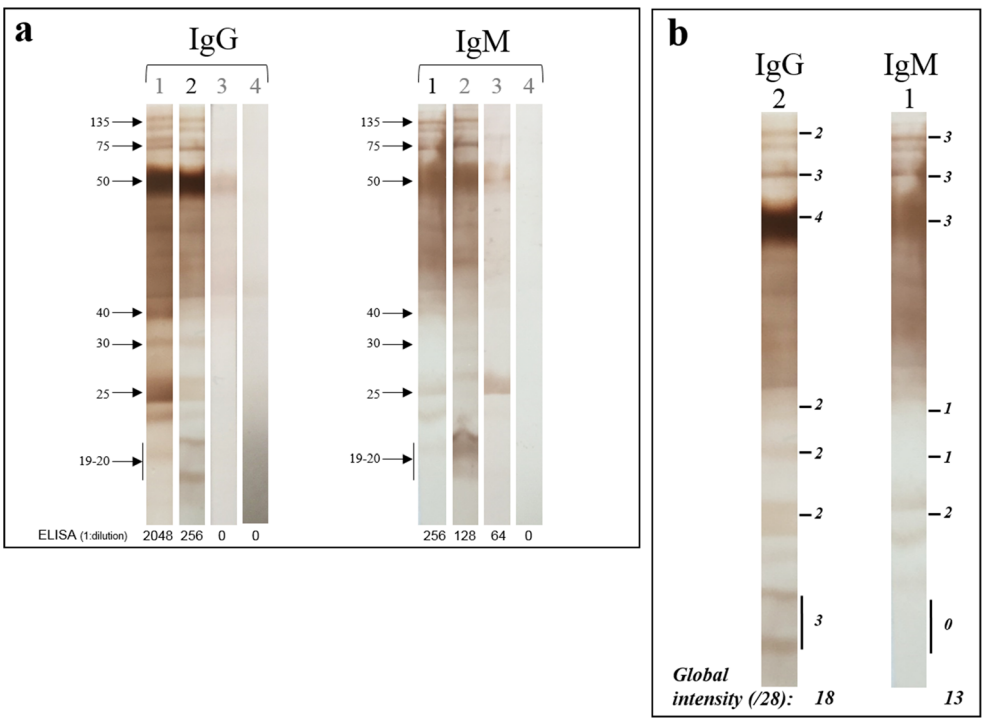

Fig. 1. Anti-*Encephalitozoon cuniculi* Western blot testing in rabbit blood samples. **a** Examples of sera showing negative and positive Western blot patterns using anti-rabbit IgG and IgM conjugates. The primary bands are highlighted by the theoretical molecular weight of the corresponding proteins indicated on the *left*. The dilution titers found in ELISA are indicated below each WB strip. **b** The WB global score is the sum of all the band intensities which range from 0 to 4 for each of the proteins located at 135, 75, 50, 40, 30, 25, and 19–20 kDa. Thus, the total is ranked on 28 points as a maximum

When evaluating all the WB bands by adding their respective intensities, the lower positive cutoff values for IgG and IgM detection were determined to be 2 and 1 out of a total of 28, while the upper values were 5 and 2 out of 28, respectively (Fig. 2). Taking into account the upper thresholds, sensitivity and specificity were estimated at 88.4 and 98.8% for anti-

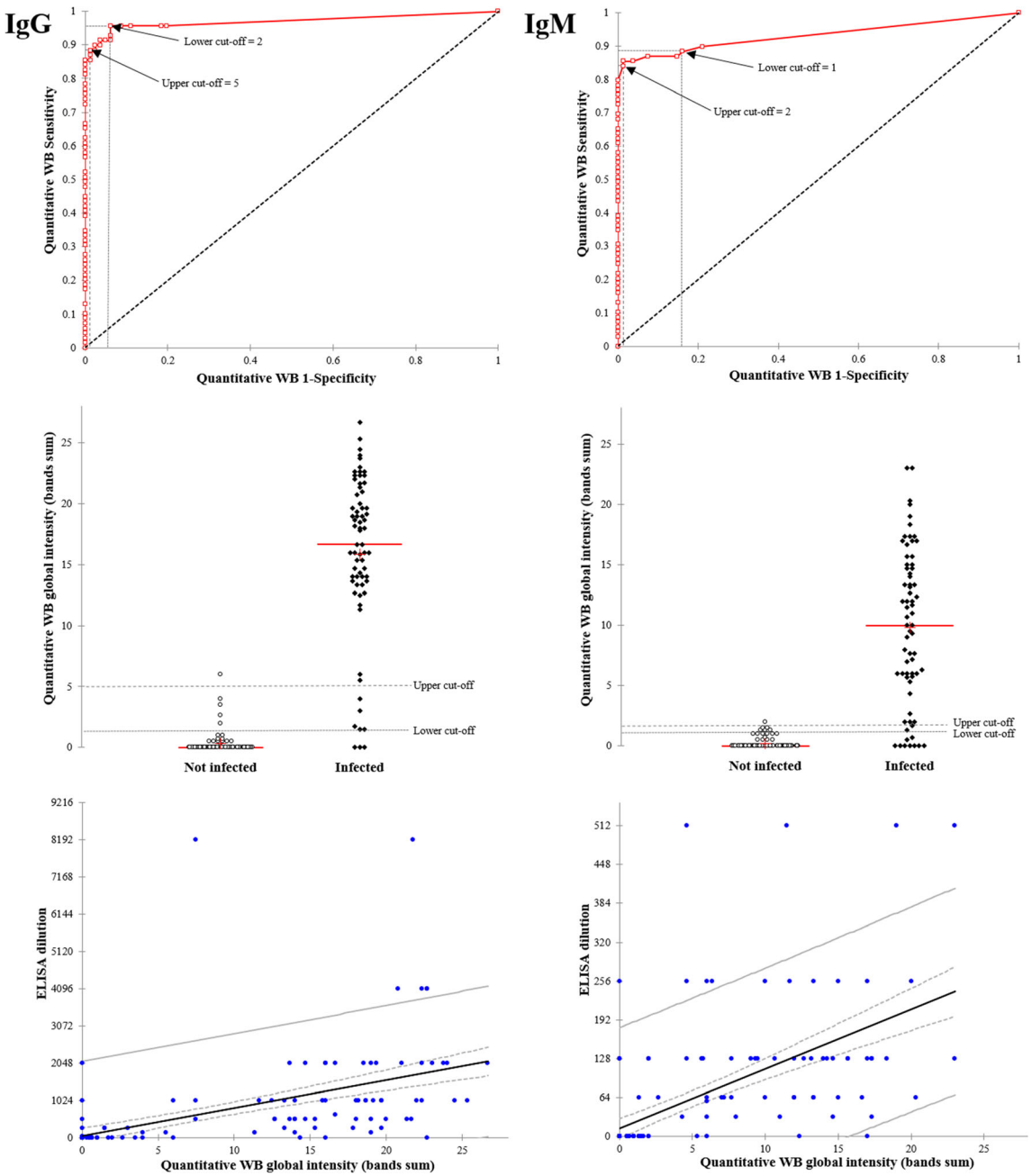

Fig. 2. Diagnostic performance of the anti-*Encephalitozoon cuniculi* quantitative Western blot testing. *Left panel*, IgG detection; *top*, the ROC curve showed AUC at 0.970, as well as sensitivity (Se) and specificity (Sp) at 91.3% [81.9–96.2%] and 93.8% [85.9–97.6%] with the lower cutoff value set at 2/28 and at 88.4% [78.4–94.2%] and 98.8% [92.5–100.0%] by the upper cutoff established at 5, respectively; *middle*, the aligned *box plot* showed distribution of the WB global scores from either side of the lower (2/28) and upper thresholds (5/28); *bottom*, the correlation between ELISA dilution and quantitative WB global intensity (band sum) was shown to be statistically high (Pearson's coefficient $R^2 = 0.307$, $P < 0.0001$) (*blue dots*, data; *solid line*, model by linear regression; *dashed line*, 95% confidence interval). *Right panel*, IgM detection; *top*, the ROC curve had an AUC estimated at 0.932, Se at 87.0% [76.7–93.1%], and Sp at 85.2% [75.6–91.4%] when the lower cutoff was set at 1/28 and Se and Sp at 98.8% [92.5–100.0%] and 84.1% [73.4–91.0%] when the positive upper cutoff value was set at 2, respectively; *middle*, the aligned *box plot* showed distribution of the WB global scores from either side of the lower (1/28) and upper thresholds (1/28); *bottom*, the correlation between ELISA dilution and quantitative WB global intensity (band sum) was shown to be significantly high (Pearson's coefficient $R^2 = 0.362$, $P < 0.0001$) (*blue dots*, data; *solid line*, model by linear regression; *dashed line*, 95% confidence interval) (color figure online)

E. cuniculi IgG and at 84.3 and 98.8% for IgM antibody detection, respectively (Table 2). Overall, when positive for both IgG and IgM detection, quantitative WB test was strongly associated with diagnosis of encephalitozoonosis (OR = 604.7, [34.6–10,566.2], $P < 0.0001$). The specificity of the combined CRP measurement with both IgG and IgM detection by quantitative WB was determined to be 100.0% [94.4–100.0], but the sensitivity decreased to 37.5% [27.2–49.1%].

Comparison of quantitative WB with ELISA

Comparative results are displayed in Table 2. Out of the rabbits with encephalitozoonosis, 76.4% samples were assessed unequivocally positive with both serology techniques for IgG detection, but 12.5% were negative in ELISA but positive in quantitative WB. Regarding IgM, the concordance rate between the two serological tests in infected rabbits was 83.3%, although 15.3% were positive only in WB and 1.4% only in ELISA.

Quantitative WB and ELISA were strongly correlated for both IgG (Pearson's coefficient $R^2 = 0.307$, $P < 0.0001$) and IgM ($R^2 = 0.362$, $P < 0.0001$) detection (Fig. 2), but respective sensitivities were better in WB (88.4 vs. 76.1%, $P = 0.008$, and 84.3 vs. 70.4%, $P = 0.009$, respectively).

Discussion

Encephalitozoon cuniculi infection continues to be difficult to diagnose in rabbits (Künzel and Joachim 2010). When subclinical, its presence may be underestimated. When clinical, signs of encephalitozoonosis must be differentiated reliably from bacterial or viral infections, injuries, or other miscellaneous inflammatory processes (Gruber et al. 2009; Künzel and Joachim 2010). Of important note is the possible zoonotic potential of this organism for immunocompromised humans and its ability to spread to other domestic species, like cats and dogs (Bywater 1979; Wasson and Peper 2000; Mathis et al. 2005). Thus, reliable diagnostic methods of *E. cuniculi* infection in rabbits, as its main domestic animal host, are of high importance.

Although antibody detection has become the preferred diagnostic tool for the *antemortem* detection of *E. cuniculi* in rabbits, some limitations of routine serology testing have prevented its reliable and satisfactory implementation in clinical settings (Künzel and Joachim 2010; Latney et al. 2014). Previous studies found no consistent IgG pattern depending on the clinical course when measured by conventional routine tests (Kunstýr et al. 1986; Csokai et al. 2009), suggesting that the observed variations may be influenced by *E. cuniculi* exposure burden and inter-individual differences in immune responses. Furthermore, IgG measurement alone was insufficient to differentiate between previous or active infection status and that routine serological tests, like ELISA or IFAT assays, do not provide reliable means to accurately study the immune response, since they do not allow differentiation between infection without symptoms and actual diseases (Jeklova et al. 2010). In a current application in the USA, quantitative titers for both IgM and IgG in ELISA format are recommended in tandem with testing for CRP (Cray et al. 2015). However, this ELISA involves a whole culture extract of *E. cuniculi*, so that antibody specificity cannot be assessed unequivocally. The use of CRP aids in determining if seropositivity is present in conjunction with systemic inflammation, but increased levels are not specific for *E. cuniculi* infection. Interestingly, the diagnostic performance of ELISA in this present study was superior to that in our previous report (Cray et al. 2015). While the methods have remained the same, this may reflect a change in the composition of the study sample set. In the current study, a larger number of samples from non-*E. cuniculi* control animals were available.

In this context, the quantitative approach of IgG and IgM detection by Western blot (WB) allows for a more precise analysis of the immune response and therefore a more reliable diagnosis (Künzel et al. 2014). Other studies involving WB

Table 2 Diagnostic performance of quantitative Western blot (WB) and enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Encephalitozoon cuniculi* antibodies in rabbit blood samples

IgG	WB positive cutoff, 5/28	ELISA positive cutoff, 1:512 dilution	*P* value	WB positive cutoff, 2 /28	ELISA positive cutoff, 1:64 dilution	*P* value
Sensitivity	88.4% [78.4–94.2]	76.1% [64.8–84.5]	0.008	84.3% [73.8–91.1]	70.4% [58.9–79.8]	0.009
Specificity	98.8% [92.5–100.0]	96.3% [89.1–99.1]	0.617	98.8% [92.5–100.0]	96.3% [89.1–99.1]	0.617
Positive predictive value	98.4% [95.3–100.0]	94.7% [88.9–100.0]	0.248	98.3% [95.1–100.0]	94.3% [88.1–100.0]	0.096
Negative predictive value	90.9% [84.9–96.9]	82.1% [74.4–89.8]	0.004	87.9% [81.2–94.6]	78.8% [70.7–86.8]	0.003
Positive likelihood ratio	71.7 [10.2–504.1]	20.5 [6.7–62.8]		68.8 [9.7–480.1]	19.0 [6.2–58.3]	
Negative likelihood ratio	0.1 [0.1–0.2]	0.2 [0.2–0.4]		0.2 [0.1–0.3]	0.3 [0.2–0.4]	

Positive cutoff values that were used for calculation are indicated directly inside the columns. In brackets are 95% confidence intervals

implementation for infectious diseases in humans, including HIV infection (Burke et al. 1987), Lyme borreliosis (Zöller et al. 1991), or chronic aspergillosis (Oliva et al. 2015), support such a usage. Besides, a previous study evaluated WB as a reference standard tool for the diagnosis of *E. cuniculi* infection in comparison with IFAT in cats (14): the two serology tests were considered comparable, but the latter was preferred for ease of use. However, quantitative assessments were not performed and the WB bands were not individually examined (Künzel et al. 2014).

In the present study, the assessment of band intensities provided greatly detailed results that allowed an improved interpretation of the results with high specificity and sensitivity. As previously reported by others (Alanio et al. 2011), higher upper cutoff values were selected to help in excluding false positives. Although the agreement for quantitative WB and ELISA was high, the former showed slightly better diagnostic performance with better sensitivity. Thus, as with ELISA but with higher NPV values, the negative WB result appears reliable enough to rule out the diagnosis of active encephalitozoonosis, even when some misleading clinical signals are present. Indeed, in models of oral or intra-tracheal experimental infection, seroconversion was quite reliable, since it could be detected early and usually preceded spore excretion by 1–2 months (Cox et al. 1979; Künzel and Joachim 2010). Another advantage of quantitative WB is that it enables a one-step technical approach for viewing the IgG and IgM response, in comparison to ELISA which requires successive twofold dilution steps for positive samples. Moreover, for the few diseased cases that we could monitor over time, quantitative WB allowed—through an increase of its global intensity score—better follow-up and detection of clinical relapse/unfavorable outcome than ELISA which appeared less subtle for such a purpose. In addition, although only few follow-up data were available, it appeared that some of the specific bands examined individually, like the one located at 135 kDa, were consistently present along the various active episodes of encephalitozoonosis (data not shown). This potential interest for the disease follow-up over time now requires further large longitudinal studies.

However, lack of standardization for the gel electrophoresis is a true limitation of WB preparation for routine use. Furthermore, the subjectivity of its interpretation—even when repeating the test in triplicate with two independent readings each—is a possible source of inter-laboratory variations that recent tools like sensitive and reproducible scanning are able to overcome (Gassmann et al. 2009). In conclusion, due to its labor-intensive and time-consuming features, quantitative WB does not appear suitable as a daily routine tool of diagnosis for *E. cuniculi* infection in rabbits. A commercial kit may support a wider use in clinical laboratories. Nonetheless, in some specialized laboratories that are already well seasoned with such techniques, it may represent a valuable approach for studying in detail the humoral response and for improved understanding of the course of pathophysiology during infection in rabbits. Further studies are required to definitively evaluate the diagnostic role of quantitative WB in such a context.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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