Comparison of assays for antibodies to *Encephalitozoon cuniculi* in rabbits


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What is This?
Comparison of assays for antibodies to
Encephalitozoon cuniculi in rabbits

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Summary

Two indirect immunofluorescence (IIF) assays, two enzyme-linked immunosorbent assays
(ELISAs) and the carbon immunoassay (CIA) for determination of antibodies to
Encephalitozoon cuniculi were compared using 210 sera of rabbits, 135 of which originated
from seven infected colonies, while 75 originated from four uninfected colonies. There was no
evidence of a difference between the different assays with respect to the number of positive
sera. There was a clear correlation between the quantitative response measured by IIF and
CIA and the other assays, and between both IIF tests, while no such correlation was found in
the quantitative response measured by ELISAs, which might be explained by the less
quantitative nature of the ELISA. Therefore quantitative determination of antibodies to E.
cuniculi should be performed by IIF and not by ELISA. The nosographic sensitivities N_t
and specificities N_s of the assays were ≥ 0.94 and ≥ 0.97 respectively. Small differences in N_t
and N_s between the assays, although not statistically significant, were responsible for
differences in the calculated predictive values of a positive test and of a negative test. As
expected, the magnitude of these differences depended on the fraction of positive sera sampled
from a given colony. There was strong evidence of such a difference between the fraction of
positive sera found in different colonies, but the sample size from some colonies was too
small to allow any conclusion, whether this was due to differences in the prevalences of the
infection in the colonies or something else. We conclude that any of the assays will be
suitable for the routine health monitoring of laboratory rabbit colonies for E. cuniculi
infection, as recommended by the Federation of European Laboratory Animal Science
Associations.

Keywords Encephalitozoon cuniculi; serology; health monitoring; rabbits

The microsporidian intracellular parasite E.
cuniculi infects a wide range of hosts
including the common small laboratory ani-
mal species and human and non-human pri-
mates (Canning & Lom 1986, Canning &
Holliester 1992). E. cuniculi has recently
emerged as an opportunist parasite in
patients infected with the human immuno-
deficiency virus (Desplazes et al. 1996).
Whether E. cuniculi is a homogeneous spe-
cies is currently unclear: phenotypic and
genotypic differences between isolates from
different hosts have been found, but generally
isolates are infective to hosts other than the
original host species upon experimental
inoculation (Didier et al. 1995).
In rabbits *E. cuniculi* infection usually causes a mild, subclinical disease, presumably affecting the animals’ immune responsiveness (Cox 1977), but the infection can also manifest itself as an overt and sometimes fatal disease (Canning & Hollister 1992, Pakes & Gerrity 1994). The Federation of European Laboratory Animal Science Associations (FELASA) recommends the periodic monitoring of laboratory colonies of rabbits for *E. cuniculi* infection [Kraft et al. 1994].

*Encephalitozoon cuniculi* infections have traditionally been diagnosed by histological examination of granulomatous lesions in brain and kidneys [Pakes & Gerrity 1994]. The application of molecular techniques (Fedorko & Hijazi 1996) to detect the parasite in laboratory animals has not been described. Immunologically based screening procedures that have been applied in rabbits consist of an intradermal delayed type hypersensitivity test [Pakes et al. 1972] and various assays to detect antibodies to the parasite. The validity of antibody assays for the detection of *E. cuniculi* infection has been evaluated by comparison with histology, and in rabbits both compared favourably [Waller 1977, Pakes et al. 1984, Greenstein et al. 1991]. Some studies have compared serological assays, e.g. CIA and IIF [Greenstein et al. 1991, Waller 1997]. The IIF has been compared with complement fixation [Pakes et al. 1984] and a microbead agglutination test [Shadduck & Geroulo 1979, Pakes et al. 1984]. Although the various serological assays were said to be equally useful, it is likely that the sample sizes were too limited to draw firm conclusions, and/or statistical analysis of the data were not appropriate.

The use of ELISA has not been reported in the monitoring of rabbits for *E. cuniculi* infection. As the latter assay was set up in both of our laboratories (RIVM and UoC), we compared our ELISAs with our IIFs and the CIA [UoC only] using rabbit sera sampled in colonies with and without previous histological evidence of *E. cuniculi* infection, in order to evaluate whether any differences between the assays with respect to their suitability for diagnosing this agent on a colony level, e.g. as recommended by the FELASA [Kraft et al. 1994], could be observed. Here the null hypothesis, defined as ‘the assays are equally applicable for health monitoring’, is tested against the alternative that ‘the assays are not equally applicable for health monitoring’.

**Materials and methods**

*Sera*

Our study was based on a sample of 135 rabbits from seven colonies in which nose-miasis had been diagnosed by histology (Table 1; infected colonies), and a sample of 75 rabbits from four colonies without such a history (uninfected colonies). In all colonies but one (K), some kind of barrier protection and health monitoring was applied. In colonies E and J breeding rabbits were periodically monitored for *E. cuniculi* infection using the ELISA performed by UoC [see below], and seropositive animals were moved to colony I. Colony K was a conventional rabbit colony which was kept without specific measures to protect the animals from infection by pathogenic microorganisms. Sera were transported in a frozen condition and stored at \(-20^\circ\text{C}\) until testing.

*Serological assays*

Testing of split samples was done by the Section of Laboratory Animal Microbiology, LIS, National Institute of Public Health and the Environment, Bilthoven, The Netherlands (RIVM), and by the Department of Experimental Medicine, The Panum Institute, University of Copenhagen and National University Hospital, Denmark (UoC).

*Indirect immunofluorescence test*

RIVM The IIF was performed as described [Boot et al. 1988]. *E. cuniculi* was propagated in RK 13 cell cultures, and spores were counted using a haemocytometer under phase-contrast and adjusted to a concentration of \(1.4 \times 10^9\) spores/ml. Volumes of 2.5 μl of the spore suspension were dropped per slide, air dried, fixed in methanol and stored at \(-20^\circ\text{C}\) before use. Slides were incubated with test sera for 45 min at 37°C, washed 3 x in PBS pH 7.2 at room temperature, stained for 30 min at 37°C with a FITC-conjugated...
Table 1 Comparison of assays for detection of antibodies to *Encephalitozoon cuniculi* in sera of rabbits from colonies with and without histological evidence of nosemiasis

<table>
<thead>
<tr>
<th>Colony status</th>
<th>Colony</th>
<th>No. of samples</th>
<th>RIVM*</th>
<th>UoC†</th>
<th>CIA</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>A</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>38</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>75</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infected</td>
<td>E</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4&lt;sup&gt;--&lt;/sup&gt;-100</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>70-90</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>12</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7&lt;sup&gt;--&lt;/sup&gt;-58</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3&lt;sup&gt;--&lt;/sup&gt;-31</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>55</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2&lt;sup&gt;--&lt;/sup&gt;-5</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>28</td>
<td>25</td>
<td>26</td>
<td>24</td>
<td>24&lt;sup&gt;--&lt;/sup&gt;86-93</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>135</td>
<td>49</td>
<td>52</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>210</td>
<td>49</td>
<td>54</td>
<td>52</td>
<td>49&lt;sup&gt;--&lt;/sup&gt;47</td>
</tr>
</tbody>
</table>

*National Institute of Public Health, NL (RIVM) and †University of Copenhagen, DK (UoC)

It was attempted to free colonies E and J from the infection by moving all ELISA positive rabbits to colony I.

IIF = indirect immunofluorescence; ELISA = enzyme-linked immunosorbent assay; CIA = carbon immunoassay

---

goat anti-rabbit Ig (Dako Ltd, Glostrup, Denmark), washed again 3 × in PBS, air dried and examined using a fluorescence microscope at 200 × magnification. Sera were screened at 1:10 dilution in PBS pH 7.2. Positive sera were re-examined using doubling dilutions up to 1:2560 in PBS.

**Enzyme-linked immunosorbent assay**

**RIVM** The *E. cuniculi* used as the antigen originated from a spontaneously infected rabbit. Stock suspension of *E. cuniculi* spores was diluted in Na<sub>2</sub>CO<sub>3</sub> buffer pH 9.6, and dilutions containing 1.4 × 10<sup>8</sup> spores/ml were coated for 24 h at 22°C to high binding polystyrene flat-bottomed microtitre plates [Greiner, Alphen aan de Rijn, The Netherlands] and plates were stored at −20°C until use. The ELISA was performed as described for *Streptobacillus moniliformis* (Boot et al. 1993): sera were diluted 1:50 in PBS pH 7.2 containing 1% BSA, and 100 μl volumes were incubated in duplicate with the antigen for 1 h at 37°C and then treated with 100 μl of peroxidase-labelled goat anti-rabbit Ig conjugate [Cappel, Malvern, USA]. Tetramethylbenzidine in DMSO was used as substrate and the reaction was stopped after 10 min by 2 mol/l H<sub>2</sub>SO<sub>4</sub>. Absorbances were read using a Titertek multiscan at 450 nm. Antigen and conjugate concentrations were pre-tested by chessboard titrations with various dilutions of the antigen and the conjugate. Uncoated control wells exposed only to substrate and stopping reagent were used to blank the reader. The ELISA was considered positive if the optical density (OD) value (extinction) of
the 1:50 serum dilution exceeded the mean + 4 SD of the mean of the ODs in the sera of rabbits from colonies without a history of *E. cuniculi* infection. The OD values of 2 samples that were evidently falsely positive by the RIVM ELISA were not included in the calculation of cut-off values.

**UoC** The ELISA was performed as previously described (Hansen & Skovgaard-Jensen 1995): antigen-coated microtitre plates (Charles River Wiga, Sulzfeld, Germany) were stored at −20°C until use. Sera were diluted 1:100 in 0.1% PBS-tween 20 and 200 µl volumes were incubated with the antigen and with uncoated wells for 2 h at room temperature, and after washing were treated with 200 µl of peroxidase-labelled goat anti-rabbit Ig conjugate (Dako Ltd, Glostrup, Denmark) for 2 h at room temperature. O-phenyldiamin was used as substrate and the reaction was stopped after 10 min by 2 mol/l H₂SO₄. Absorbances were read at 492 nm using a Sigma microplate reader (Sigma, St Louis, USA). Antigen and conjugate concentrations were pre-tested by chessboard titrations with various dilutions of the antigen and the conjugate. Uncoated control wells exposed only to substrate and stopping reagent, were used to blank the reader. The ELISA was considered positive if both the difference between the ODs of the coated and uncoated well was more than 0.2, and if the OD of the coated well was more than 0.394 (the mean + 3 SD of a range of sera of rabbits from colonies without a history of *E. cuniculi* infection).

**Carbon immunoassay**
The assay was performed as described (Waller 1977) using antigen (Testman, Uppsala, Sweden), coated onto slides, air dried, flame fixed and stored at −20°C until use. Sera were screened at 1:20 dilution in PBS pH 7.2. Five microlitre of the diluted serum was mixed with 5 µl India ink (Testman, Uppsala, Sweden) and left on the coated slide for 5 min. Slides were then washed with PBS to remove all ink and were finally examined for agglutination of spores using a light microscope at 400× magnification. Positive sera were re-examined using doubling dilutions of up to 1:2560 in PBS.

In all assays (ELISA, IIF and CIA) negative and positive control sera (the latter obtained from rabbits immunized with spores of *E. cuniculi*) were run in each test.

**Evaluation of data/statistical analysis**
Differences in the numbers of positive samples obtained using the assays and in the numbers of seropositive rabbits between colonies were analysed using the Chi-square test. Multiple correlation coefficients of the correlation of the quantitative response of each assay as compared with the other tests, as well as correlation coefficients of the correlation of the quantitative response of RIVM ELISA to UoC ELISA, and RIVM IIF to UoC IIF were calculated. The sensitivity and specificity of the assays were calculated as the nosographic sensitivity ($N_1$) and the nosographic specificity ($N_2$), respectively. $N_1$ is defined as the proportion of infected rabbits reacting truly positive (TP) in the test and $N_2$ as the proportion of uninfected rabbits reacting truly negative (TN) in the test (Hansen 1993). In order to estimate $N_1$ and $N_2$, serum samples were considered TP or TN upon showing three positive or negative results, respectively, after testing by the five assays. Differences in the sensitivity and specificity of the assays were evaluated by the Chi-square test. The predictive values for a positive test ($PV+\uparrow$) and a negative test ($PV-\downarrow$) were calculated according to Jacobson and Romatowski (1996):

\[
PV+ = \frac{P \times N_1}{P \times N_1 + (1 - P) \times (1 - N_2)}
\]

\[
PV- = \frac{(1 - P) \times N_2}{(1 - P) \times N_2 + P \times (1 - N_1)}
\]

in which $P$ = prevalence of infection (% of positive sera), $N_1$ = nosographic sensitivity and $N_2$ = nosographic specificity of the assay.

**Results**
Table 1 shows the outcome of determinations of antibodies in rabbit sera to *E. cuniculi* using IIF, ELISA and CIA. Sera of rabbits from the four colonies not having a history of...
**Table 2** Chi-square tests for comparing differences in number of positive samples between colonies and between assays

<table>
<thead>
<tr>
<th>Colony</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>4</td>
<td>10</td>
<td>12</td>
<td>16</td>
<td>55</td>
<td>28</td>
<td>125</td>
</tr>
<tr>
<td>Positive observed</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>25</td>
<td>49</td>
</tr>
<tr>
<td>Expected</td>
<td>1.57</td>
<td>3.92</td>
<td>4.70</td>
<td>6.27</td>
<td>21.56</td>
<td>10.98</td>
<td></td>
</tr>
<tr>
<td>Negative observed</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>13</td>
<td>53</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td>Expected</td>
<td>2.43</td>
<td>6.08</td>
<td>7.30</td>
<td>9.73</td>
<td>33.44</td>
<td>17.02</td>
<td></td>
</tr>
</tbody>
</table>

$\chi^2 = 76.5; df = 5; P = 0.000$

**Table 3** Differences in test results in 12 samples of rabbits from *Encephalitozoon cuniculi* infected colonies using various antibody assays

<table>
<thead>
<tr>
<th>Colony</th>
<th>Sample</th>
<th>RIVM</th>
<th>UoC</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>9</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

$\chi^2 = 0.63; df = 4; P = 0.96$. See Table 1 for abbreviations.

*E. cuniculi* infection were negative in all assays, except two (2.7%) that were clearly positive in the RIVM ELISA (Table 1). All assays detected positive sera in six of the seven colonies with previous histological evidence of *E. cuniculi* infection. Applying the $\chi^2$ test (Table 2) there is strong evidence of a difference between the fraction of positive sera found in the different colonies, while there is no evidence of a difference in the number of positive samples detected by the different assays.

In 12 of the 135 (8.9%) sera from infected colonies the outcome of the assays disagreed (Table 3), but no systematic differences were found and all assays, except the RIVM IIF (see below), yielded one or more apparently aberrant results.

Table 4 shows the predictive values of a positive test ($PV^+$) and a negative test ($PV^-$) calculated from $N_1$ and $N_2$ for the assays, in colonies showing (arbitrarily) high, intermediate and low prevalences. The $PV^+$ and $PV^-$ were all above 93% in colonies showing an intermediate (30%) or high (60%) prevalence of infection. With low prevalences (10 to 1%) the $PV^-$ almost invariably increased to 100% but the $PV^+$ values decreased for most assays, with the exception of the RIVM IIF (Table 4). The nosographic sensitivity ($N_1$) of the assays (Table 4) was at least 0.94 and the IIF performed by UoC showed maximal sensitivity.
Table 4: The nosographic sensitivity ($N_1$)* and specificity ($N_2$) of five antibody assays for detection of *Encephalitozoon cuniculi* infection in rabbits and calculated predictive values of positive (PV+) and a negative test (PV-) at some assumed prevalences.

<table>
<thead>
<tr>
<th></th>
<th>RIVM</th>
<th>UoC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIF ELISA</td>
<td>IIF ELISA &amp; CIA</td>
</tr>
<tr>
<td>Sensitivity ($N_1$)*</td>
<td>0.98</td>
<td>1.0</td>
</tr>
<tr>
<td>Specificity ($N_2$)</td>
<td>1.0</td>
<td>0.99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prevalence %</th>
<th>PV+</th>
<th>PV-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RIVM</td>
<td>UoC</td>
</tr>
<tr>
<td></td>
<td>IIF ELISA</td>
<td>IIF ELISA &amp; CIA</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>98.0</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>93.3</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>78.4</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>24.8</td>
</tr>
<tr>
<td>60</td>
<td>97.1</td>
<td>97.0</td>
</tr>
<tr>
<td>30</td>
<td>99.2</td>
<td>99.1</td>
</tr>
<tr>
<td>10</td>
<td>99.8</td>
<td>99.8</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>99.9</td>
</tr>
</tbody>
</table>

*$N_1$ and $N_2$ are the proportions of infected rabbits reacting positive or negative respectively in $\geq 3$ assays. See Table 1 for abbreviations.

The nosographic specificity ($N_2$) was at least 0.97 and the IIF performed by RIVM showed maximal specificity (1.0). There were no statistically significant differences in the sensitivity or specificity of the assays ($\chi^2$ test, $pN_1 > 0.5$, $pN_2 > 0.25$).

Table 5 shows the multiple correlation coefficients of the quantitative response measured by each test as compared with the other tests, while Table 6 shows the correlation coefficients of the quantitative outcome of RIVM ELISA to UoC ELISA, as well as RIVM IIF to UoC IIF. No correlation was found in the antibody levels measured by ELISAs, neither when correlated to the other tests nor to one another, while there was clear correlation between the quantitative outcome of IIF and CIA and the other tests, and between the two IIF tests.

### Discussion

The overall results of serological monitoring of the rabbit colonies (Table 1) agreed very well with their history. Those four colonies without previous histological evidence of *E. cuniculi* infection (considered as uninfected) were seronegative in all but one assay. It is unclear why two sera from two uninfected colonies were seropositive in all but one assay. It is unclear why two sera from two uninfected colonies were clearly positive in the RIVM ELISA (Table 1). All but one of the seven infected colonies showed seropositive animals in all assays. Colony E appeared negative upon testing, suggesting that systematic

---

Table 5: Multiple correlation coefficients of the correlation of antibody levels to *Encephalitozoon cuniculi* measured by each of five different assays as compared with the other assays (49 samples)

<table>
<thead>
<tr>
<th></th>
<th>RIVM</th>
<th>UoC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA IIF</td>
<td>ELISA IIF &amp; CIA</td>
</tr>
<tr>
<td>Multiple c.c. adjusted for degrees of freedom (%</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>P value*</td>
<td>0.331</td>
<td>0.000</td>
</tr>
<tr>
<td>Power</td>
<td>0.0512</td>
<td>0.9671</td>
</tr>
</tbody>
</table>

*Analysis of variance, using $h_0: D = 0$. See Table 1 for abbreviations.

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removing of all ELISA-positive breeders had freed the colony from infection. Although the periodic testing of all animals and the removing of all positive responders has been claimed to be successful in the eradication of *E. cuniculi* infection from rabbit colonies (Cox et al. 1977, Bywater & Kellett 1978, Waller 1998), the number of samples in this colony, however, was too small to draw firm conclusions about its infection status.

It must be kept in mind that sampling eight animals from a colony (as is recommended by the FELASA [Kraft et al. 1994]) will only allow detection of infections if they are present in about 30% of the animals [with 95% probability] and prevalences of less than 30% seem to be common in rabbits [Waller 1988]. In this study, there is strong evidence of a difference between the number of positive sera found in the different colonies (Table 2). Whether this is due to the fact that there really is a difference in prevalence of the infection or something else cannot be stated, due to the small sample size of some colonies. There is no evidence of a difference in the number of positive sera detected by the different assays [Table 2]. A sample size of at least 3100 would have been necessary to detect a difference between proportions as close as those observed when the power is set to 0.90. However, such small differences are of no importance for the application of these assays in routine health monitoring.

It is not clear why 12 samples from infected colonies showed different outcomes in the assays [Table 3]. Differences in the outcome of IIFs and ELISAs vs CIA may be due to the fact that IIF and ELISA measure IgM and IgG [due to the specificity of the conjugates used], whereas the CIA measures 7S IgG antibody only [Waller et al. 1978]. If the *E. cuniculi* infection exists for some time, IgG antibodies will develop together with histological lesions, and it is therefore not surprising that histologically positive rabbits were invariably seropositive by IIF and CIA [Waller 1977, Greenstein et al. 1991]. Inherent to our study the time of infection of the rabbits is unknown. The data given by Pakes et al. (1984) cannot be compared with ours as their sample size was very limited and part of the samples were pooled sera. Recent infection with IgM antibodies only, may have been present in the CIA-negative rabbits that were positive in at least one of the other assays. Ig class differences, however, cannot explain a CIA-positive outcome in a sample that was negative in all other assays nor can they explain variable results in the IIFs and ELISAs [Table 3]. Differences between the assays may have occurred due to differences in the antigen. However, studying these 12 samples with a non-uniform outcome, the UoC–IIF and the UoC–CIA, which are based upon the same antigen, are inconsistent with the other tests, and therefore there is no reason to believe that the differences are due to differences in the antigen.

If the quantitative response measured by the ELISAs is correlated to the other tests or to one another, the correlation is too small to be of any interest [Tables 5 and 6]. To be of any interest the correlation should be more than 50%, which could have been shown in the sample size of 49 with a power of more than 0.90, but no such correlation was found. There is, however, correlation between the quantitative response measured by IIF and CIA and the other tests and between the two IIF tests [Tables 5 and 6]. This may be explained by the fact that IIF and CIA titres are determined in serially diluted sera, i.e. there is no upper limit in contrast to ELISA where an upper limit is determined by the quantity of antigen coated to the well. When there is no more antigen available for antibody to react with, the washing step will remove excess antibody. Therefore, quantitative examinations should be performed by IIF and not by ELISA.

No serological assay can identify with absolute certainty which rabbit is infected. In order to compare the sensitivity and the spe-
cificity of the assays we considered a serum to be true positive (TP) or true negative (TN) upon showing three positive or negative results respectively using the five assays. Subsequently we calculated the nosographic sensitivity (\( N_1 \)) defined as the proportion of infected rabbits reacting as TP in the test, and the nosographic specificity (\( N_2 \)) as the proportion of uninfected rabbits reacting as TN in the test (Hansen 1993). This approach did not reveal that any differences in the sensibility and specificity of the assays were of any interest in practice (Table 4).

As expected, the nosographic sensitivity (\( N_1 \)) and specificity (\( N_2 \)) of the assays determined the predictive values of a positive test (\( PV^+ \)) as well as that of a negative test (\( PV^- \)) (Table 4). As the prevalence of infection (or the % of seropositives) drops, the \( PV^+ \) increases to very high levels, even for assays with moderate \( N_1 \) and \( N_2 \) values (Tyler & Cullor 1994). Simultaneously the \( PV^- \) falls precipitously for assays in which the specificity (\( N_2 \)) is not maximal (1.0); this was the case with all assays with the exception of the RIVM IIF. Given their high sensitivity, all assays seem to be suitable for screening rabbit colonies for \( E. cuniculi \) infection. Positive test results obtained with assays other than the RIVM IIF might be confirmed by the latter assay, as its specificity appeared maximal, so all its positive results might be trusted. It should be mentioned that the method applied for the determination of true and false positives implies that some of the calculated specificities might be too low as all false positives—except those two found by the RIVM ELISA—were found in colonies in which infection was actually present.

A larger sample might have resulted in a statistically significant difference between the specificities of e.g the RIVM IIF and the RIVM ELISA or the UoC IIF and the UoC ELISA/CIA. However, a specificity of 0.97 is acceptable for an assay applied in laboratory animal health monitoring, and a lower sensitivity can be fully compensated by sampling more animals in a random sample for routine monitoring (Hansen 1993). We therefore conclude that IIF, ELISA and CIA are all suitable for the routine health monitoring of laboratory rabbit colonies for \( E. cuniculi \) infection as recommended by FELASA (Kraft et al. 1994). The occurrence of small differences in sensitivity and specificity of the assays performed in different laboratories must however be kept in mind, as they may have a considerable impact on the predictive value of both positive and negative test results in colonies with a low prevalence of infection.

References


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Encephalitozoon cuniculi antibody assays


