Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis

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Abstract

Affinity-purified 38 kDa surface antigen of Neospora caninum tachyzoites was used to develop an enzyme-linked immunosorbent assay (ELISA) to diagnose N. caninum-associated abortion in cattle. The specificity of this antigen was demonstrated by using sera from cattle experimentally infected with other apicomplexan parasites. In a panel of field sera, serological differences could be demonstrated between herds with epidemic and endemic abortions. Optimal ELISA cut-offs were determined for the detection of association between seropositivity and abortion in herds with N. caninum-associated abortions. These optimal cut-offs differed markedly when only sera from either endemic or epidemic cases were used for cut-off determination. It may thus be appropriate to apply different cut-offs in serological tests depending on the abortion pattern to be analysed.

1. Introduction

Neospora caninum is a frequently diagnosed cause of epidemic and endemic bovine abortion worldwide [1]. The dog has been identified as a definitive host of N. caninum [2,3]. Vertical transmission or propagation seems to be of major importance in the spread of N. caninum and most congenital infections result in the birth of apparently healthy calves [4–6].

In addition to histological and immunohistochemical techniques or PCR used to detect parasite antigens or DNA in aborted foetal material [1,7], N. caninum abortions in cattle herds can be diagnosed by demonstrating antibodies and statistical association between seropositivity of the dams and abortion [8]. Serological tests for the specific detection of antibodies to N. caninum include indirect fluorescent antibody test (IFAT) [1,7,9], immunoblotting [10,11], and several enzyme-linked immunosorbent assays (ELISAs) [9,10].

A requirement for a test meant to detect association between seropositivity and abortion is its ability to discriminate among the dams at risk between infected animals aborting due to N. caninum and non-aborting cattle. This seems to be possible for many assays simply by careful cut-off selection, since in a number of serological assays N. caninum-infected aborting dams tend to have higher antibody levels as compared with the non-aborting infected dams [10,12–15].

Surface antigens of apicomplexan parasites are often immunodominant and seem to be of particular interest for diagnostic purposes [11,16]. Two of these surface antigens (Nc-p43, Nc-p36) have recently been cloned [16–18] but as yet no results have been published on the potential diagnostic use of these antigens. However, serological tests based on a single, well defined immunodominant surface antigen might be superior in terms of repeatability, reproducibility and specificity compared to assays based on more or less defined antigen mixtures. Therefore we selected p38 as an...
immunodominant surface antigen of \textit{N. caninum} tachyzoites [19], which is identical to Nc-p43 (Hemphill and Schares, unpublished), to evaluate the diagnostic potential of this antigen. Using p38, we developed a diagnostic ELISA for \textit{N. caninum}-associated bovine abortions, and determined optimal cut-offs for the use of this ELISA in herds with epidemic and endemic abortions.

2. Material and methods

2.1. Parasites

The NC-1 strain of \textit{N. caninum} [20] was maintained as previously described [10]. Tachyzoites were used immediately for IFAT or frozen at \(-80^\circ\text{C}\) until used for immunoblot or ELISA.

2.2. Sera

To evaluate the ELISA, sera from cattle experimentally infected with \textit{N. caninum} (\(n = 3\)), \textit{Toxoplasma gondii} (\(n = 10\)), \textit{Sarcocystis cruzi} (\(n = 7\)), \textit{Sarcocystis hominis} (\(n = 2\)), \textit{Babesia divergens} (\(n = 10\)), \textit{Cryptosporidium parvum} (\(n = 3\)) and \textit{Eimeria bovis} (\(n = 2\)) were used (for details, see Ref. [10]).

Field sera were collected from cattle (\(n = 827\)) out of herds with abortion problems. Herds with and without \textit{N. caninum}-associated abortions were selected according to the criteria described in a previous study [10]. Briefly, abortions were regarded as \textit{N. caninum}-associated if, among the animals at risk, the abortions could be statistically associated with a positive \textit{N. caninum} serology (positive IFAT cut-off 1:50). \textit{N. caninum}-associated abortion series were further classified into endemic and epidemic abortion (for definition, see Ref. [10]).

The majority of sera used in the present study had previously been analysed to evaluate a total lysate based \textit{N. caninum} ELISA. They were collected in herds 1–11 (for a detailed description of the herds, see Ref. [10]). In addition to these samples, sera from cattle (\(n = 371\)) out of three other herds with abortion problems were analysed in the present study. One herd (herd 12) had experienced an abortion epidemic, during which 14 cows (25\% of dams at risk) aborted within 43 days. In this herd blood was collected 57–297 days after the abortions. Among the animals at risk, abortions were associated significantly (Fisher exact test; \(P = 0.0165\)) with a positive \textit{N. caninum} IFAT result (positive IFAT cut-off 1:50).

In herd 14, seven cows (12.5\% of cows at risk) aborted. Blood was collected 57–297 days after the abortions. Among the animals at risk, abortions were associated significantly (Fisher exact test; \(P = 0.038\)) with a positive IFAT result (positive IFAT cut-off 1:50).

All field sera were classified in ‘gold standard’-positive (POS\textsubscript{GS}-sera) and ‘gold standard’-negative (NEG\textsubscript{GS}-sera) using IFAT and immunoblot results to discriminate between \textit{N. caninum}-infected and non-infected cattle [10]. Briefly, sera positive in IFAT (positive cut-off 1:25) and in immunoblot (at least two immunodominant antigens recognised) were defined as POS\textsubscript{GS}-sera. Sera negative in one of the tests or negative in both tests were regarded as NEG\textsubscript{GS}-sera. Comparisons of aborting and non-aborting cows in herds with \textit{N. caninum}-associated abortions were restricted to the animals which were at least 2 years old (Table 1). Analyses regarding differences between aborting and non-aborting cows in herds with epidemic abortion pattern were restricted to POS\textsubscript{GS}-sera from the animals at risk (i.e. animals that had been pregnant for 58–260 days at the beginning of the epidemic abortion episode) (Table 1). Similar analyses in herds with an endemic abortion pattern were restricted to POS\textsubscript{GS}-sera from cattle of at least 2 years of age (i.e. the animals at risk in these herds).

2.3. IFAT, immunoblot

For IFAT and immunoblot, cell culture-derived \textit{N. caninum} NC-1 [20] tachyzoites were used. The IFAT was performed as described [6,10]. Serum dilution started at 1:25.

In the immunoblot, reactivity of the sera with immunodominant tachyzoite antigens of 17, 29, 30, 33 and 37 kDa was recorded [10].

2.4. Affinity purification of surface antigen p38

Affinity-purified p38 surface antigen was produced by using the mouse monoclonal antibody (mAb) 4.15.15 (IgG2a) coupled to rProtein A Sepharose 4B (Pharmacia & Upjohn, Uppsala, Sweden).

Table 1

<table>
<thead>
<tr>
<th>Type of abortion</th>
<th>Aborting</th>
<th>Non-aborting</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>POS\textsubscript{GS}</td>
<td>NEG\textsubscript{GS}</td>
<td>POS\textsubscript{GS}</td>
<td>NEG\textsubscript{GS}</td>
</tr>
<tr>
<td>Epidemic cases</td>
<td>26 (26)</td>
<td>4 (3)(^a)</td>
<td>96 (38)</td>
</tr>
<tr>
<td>Endemic cases</td>
<td>25 (25)</td>
<td>9 (9)</td>
<td>43 (43)</td>
</tr>
<tr>
<td>Total</td>
<td>51 (51)</td>
<td>13 (12)</td>
<td>139 (81)</td>
</tr>
</tbody>
</table>

\(^a\) POS\textsubscript{GS}, positive; NEG\textsubscript{GS}, negative. Number of animals at risk are given in parentheses.

\(^b\) One of the NEG\textsubscript{GS} dams had aborted three months prior to the abortion epidemic and therefore was not regarded as ‘at risk’.
Biotech) [21]. mAb 4.15.15 recognises an epitope on a p38 surface antigen of *N. caninum* tachyzoites, previously characterised by mAb 5.2.15 [19]. We used mAb 4.15.15 since it binds more effectively to rProtein A Sepharose 4B than mAb 5.2.15 (Scharres, unpublished).

Prior to affinity purification, cell-culture derived *N. caninum* tachyzoites were extracted in phosphate-buffered saline (PBS), 0.5% Triton X-100 (4°C). After sonication on ice for 90 s (50% active cycle, output control level 2; VibraCell), the suspension was centrifuged at 13 000 g for 10 min 4°C. The supernatant was applied to the immunosorbent which had been equilibrated with PBS, 0.1% Triton X-100. After 2 h the immunosorbent was washed with 100 volumes PBS, 0.1% Triton and eluted with 0.1 M glycine, pH 2.6. The eluent was neutralised using 1 M Tris and 10× PBS. The purity of p38 was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting and india ink staining [19].

### 2.5. ELISA

The affinity-purified antigen was diluted in coating buffer (0.1 M sodium bicarbonate, pH 8.3) and used to sensitise ELISA plates (Nunc-Immuino (Polysorb)) at 37°C for 1 h. Wells were then washed three times with PBS-T (PBS, pH 7.2, 0.05% Tween-20) and incubated with blocking solution (PBS-T, 2% horse serum) at 37°C for 0.5 h. Wells were then emptied and the serum samples (diluted 1:200 in PBS-T, 2% horse serum) added. A post-infection serum from heifer 44 was used as a positive control and a pre-infection serum from cow 24 served as a negative control [10]. After serum incubation and washing, a biotinylated monoclonal anti-bovine IgG (Sigma) diluted 1:2000 was added to the wells (37°C, 30 min) followed by washing and incubation with an extravidin-peroxidase conjugate (Sigma) diluted 1:4000. All conjugate dilutions were prepared in PBS-T, 2% horse serum. After each step, the wells were washed three times with PBS-T. After the final step, the plates were washed three times with PBS-T and twice with distilled water. Bound antibodies were detected by incubation with a substrate containing 100 μg/ml 3,3′,5,5′-tetramethylbenzidine and 0.004% hydrogen peroxide in 0.2 M sodium acetate and 0.2 M citric acid at 37°C. The reaction was stopped after 15 min by adding sulfuric acid to a final concentration of 2 N, and optical density (OD) values were measured at 492 nm (reference filter: 620 nm) on a microplate reader (SLT Labinstruments).

By checkerboard titrations, optimal assay conditions, especially the serum dilution of 1:200, were established for the purpose of the test.

Sample index values were recorded as the arithmetic mean of two index values, SI1 and SI2. These index values were calculated by the formula $SI_n = (S_n - N)/(P - N)$ where SI is one of the two individual index values, and $S_n$ one of the two individual OD values obtained for a single sample. $N$ is the arithmetic mean of two OD values obtained for the negative serum, and $P$ represents the arithmetic mean of two OD values obtained for the positive serum.

### 2.6. Computing and statistics

In the case of dichotomous variables, non-parametric differences between groups were statistically analysed by the Fisher exact test (Statistica 5.0). Since ELISA and IFAT results were not normally distributed, the Mann–Whitney *U*-test (Statistica 5.0) was employed to analyse non-parametric distribution differences of variables between groups. In all statistical analyses, the α-level of significance was set to 0.05. IFAT titres were logarithmically (log_{10}) transformed for statistical analysis. To describe sensitivity and specificity of the newly developed ELISA as a function of a selected cut-off, the two-graph receiver operating characteristic was analysed by employing a computer program TG-ROC [22]. ELISA sensitivity and specificity were estimated using optimum cut-offs determined by TG-ROC, and 95% confidence intervals (CI) were computed using a normal approximation [23].

### 3. Results

#### 3.1. Affinity purification of surface antigen p38

Affinity purification of p38 resulted in an antigen that was almost free from contaminating components (Fig. 1). In some preparations a high $M_r$ compound was visible (>94 kDa). These preparations were not used in this study.

#### 3.2. Antibody responses of dams experimentally infected with *N. caninum*

The antibody profiles as determined by IFAT [10] were compared with those obtained with the p38-ELISA for animals experimentally infected with *N. caninum* (Fig. 2a–c).

IFAT titres in the three cattle increased from 4–14 days p.i. and peaked 14–91 days p.i. In ELISA, the three cattle showed an initial increase in indices starting 9–14 days p.i. The ELISA indices slowly increased over time in all three animals until the end of the observation period. Heifers 44 and 49 in addition showed one and two spikes, respectively, (sharp increases and subsequent drops) in the ELISA indices that could temporally be associated with abortions (both animals) and a subsequent pregnancy (heifer 49) (Fig. 2b,c).

#### 3.3. Comparison of ELISA, IFAT, and immunoblot results

Mean ELISA indices of sera ($n = 827$) grouped according to their IFAT results increased with increasing IFAT titres (Fig. 3a). The ELISA indices of the sera with an IFAT titre of $<1:25$ were significantly different from the ELISA indices observed in all groups of sera with IFAT titres $\geq 1:25$ ($P < 0.001$, Mann–Whitney *U*-test). A similar observation was made when the sera were
grouped according to the number of recognised immunodominant bands (17, 29, 30, 33 kDa, and 37 kDa). The mean ELISA indices increased with increasing numbers of recognised bands (Fig. 3b). The ELISA indices of groups of sera recognising one or more of the five bands differed significantly ($P < 0.001$, Mann–Whitney $U$-test) from the ELISA indices of the serum group recognising none of the bands.

3.4. Analytical specificity of the ELISA

Sera from animals experimentally infected with *T. gondii*, *S. cruzi*, *S. hirsuta*, *S. hominis*, *C. parvum*, *B. divergens*, and *E. bovis* yielded ELISA indices which were not significantly higher than those obtained with IFAT/immunoblot-negative (NEGGS) field sera (Fig. 4). Within the group of dams older than 2 years ($n = 573$), no significant differences were observed between the ELISA indices of NEGGS sera collected from aborting ($n = 13$) and non-aborting ($n = 305$) dams in herds with *N. caninum*-associated abortions and from dams in herds without *N. caninum*-associated abortions ($n = 65$) (Fig. 4).

3.5. Differences between aborting and non-aborting dams

The analysis of field sera from herds with *N. caninum*-associated abortions was restricted to dams ≥ 2 years ($n = 190$). Within the group of POSGS dams, the aborting animals ($n = 51$) had significantly higher ELISA indices than the non-aborting cattle ($n = 139$) ($P < 0.001$, Mann–Whitney $U$-test) (Fig. 4).

To compare aborting with non-aborting POSGS dams, the analysis was restricted to the group of animals at risk. When dams at risk in herds with endemic abortions were compared with dams at risk from herds with epidemic abortions, the latter animals had significantly lower ELISA indices ($P = 0.0002$; Fig. 5 and Table 2). By IFAT, the differences between the dams from herds with epidemic and endemic...
abortions was opposite: animals from herds with epidemic abortions had significantly higher IFAT titres than those from herds with endemic abortions ($P < 0.0001$; Fig. 5 and Table 2).

The situation was similar when herds with epidemic and endemic abortions were compared within the group of the aborting and non-aborting dams (Fig. 5 and Table 2). However, in the case of the aborting dams, ELISA indices tended to be higher in endemic than in epidemic herds, but the differences were not statistically significant ($P = 0.059$; Fig. 5 and Table 2).

Among the POS$_{GS}$ dams at risk from herds with epidemic and endemic abortions, aborting dams had higher ELISA indices or IFAT titres as compared to non-aborting dams (Fig. 5 and Table 2). These differences were significant except for the ELISA indices of aborting and non-aborting dams from endemic herds (Table 2).

### 3.6. TG-ROC analyses

To establish the cut-offs for the p38-ELISA that optimally discriminate between infected aborting and non-aborting dams, TG-ROC analyses were performed using sera from dams at risk (n = 396, Table 1) as ‘gold standard’ positive or negative. Sera from POS$_{GS}$ dams at risk that had aborted were regarded as ‘gold standard’-positive. All other dams at risk (NEG$_{GS}$ dams that aborted and all non-aborting dams) were considered as ‘gold standard’-negative. The optimal cut-off determined for the p38-ELISA using sera from epidemic and endemic herds was $d_0 = 0.153$ (Fig. 6a). At
this cut-off the ELISA discriminates POS_GS dams at risk from other dams at risk with an equal sensitivity and specificity ($\theta_0$) of 83% (Fig. 6a and Table 3). Restriction to the epidemic herds led to a lower cut-off in the p38-ELISA as compared to the situation when sera from both, endemic and epidemic herds were used (Fig. 6b and Table 3). When the analysis was restricted to endemic herds, the optimal cut-off of the p38-ELISA was higher as compared with the cut-offs determined when sera from both, endemic and epidemic herds were used (Fig. 6c and Table 3).

4. Discussion

In the present study, an affinity-purified surface antigen, p38, of N. caninum tachyzoites has been applied to develop an ELISA to diagnose N. caninum-associated abortions. p38 and the major surface antigen Nc-p43/NcSRS-2, described and cloned by others [16–18,24], are identical (Hemphill and Schares, unpublished). Conformational epitopes seem to play an important role for the immunogenicity of p38, since reactivities of bovine infection sera against this antigen are markedly lowered when the antigen is reduced by $\beta$-mercaptoethanol (Scharres, unpublished). Since in recombinant proteins the conformation and post-translational modifications may be different as compared to the N. caninum-derived protein, we used the latter rather than recombinant p38 antigen for our study.

Although the gene encoding Nc-p43 reveals sequence homologies to the SRS-2 gene of T. gondii [16,17], cross reactivities of this antigen with T. gondii infection sera seem to be negligible as determined by immunoblotting [25,26]. Our study confirms these observations. Moreover, the results obtained with sera from cattle infected with other apicomplexan parasites (Sarcocystis spp., C. parvum, E. bovis, B. divergens) demonstrate the specificity of this particular antigen.

Together with Nc-p36/NcSAG1, p38/Nc-p43/NcSRS-2 is an antigen commonly detected by infection sera [11,25,26]. This observation, the specificity of the reaction with p38, and the immunodominant nature of the antigen suggest that p38 is an ideal candidate for the development of sensitive and specific serological tests.

For the diagnosis of bovine neosporosis, serological tests are required to determine N. caninum as a likely cause of abortion or to analyse vertical propagation of the parasite [11]. Since p38 seems to be present only in tachyzoites but not in bradyzoites [19], it might also be a good candidate for developing a diagnostic test which allows to detect antibody reactions in the acute phase of the infection or after recrudescence. Both events may cause abortion in pregnant dams. The expectation regarding the performance of the p38-ELISA in detecting acute infection was met by the antibody responses two pregnant heifers experimentally infected with N. caninum developed against the antigen, since their antibody levels against p38 peaked after infection and prior to abortion. A second peak, later observed in one of the heifers,
could possibly reflect a recrudescence of the infection in this animal. However, in a third dam (which was not pregnant) the antibody response against p38 did not peak after infection but increased slowly. This shows that the time course of the antibody response against p38 can be variable in individual dams. The reasons (e.g. pregnancy, partial immunity) leading to these differences remain to be identified in further studies. Individual time courses of *N. caninum* specific antibodies in pregnant dams have also been observed by others [27,28] and time course characteristics predictive for the outcome of the pregnancy regarding vertical transmission and abortion were identified [27].

A diagnostic test used to examine herds with abortion problems for statistical association between abortion and seropositivity requires to differentiate among the dams at risk between (i) dams which have aborted due to the infection, and (ii) the remaining dams which have not aborted. In the present study we showed that cattle aborting due to neosporosis possess higher antibody levels against p38 than non-aborting dams. Although the same observation has been made in a number of earlier studies using other assays [10,12–15], the present study is the first one which employs this observation directly for selecting appropriate diagnostic cut-offs. Using TG-ROC, cut-offs were computed at which the ELISA optimally discriminated between infected aborting and other dams at risk using sera from herds with *N. caninum*-associated epidemic and endemic abortions.

### Table 3

<table>
<thead>
<tr>
<th>Herds analysed</th>
<th>ELISA</th>
<th>'Gold standard'</th>
<th>(\theta_u(%))</th>
<th>% Sens. 95% CI</th>
<th>% Spec. 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos.</td>
<td>Neg.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (0.153)</td>
<td>42</td>
<td>57</td>
<td>83</td>
<td>68.6–91.1</td>
<td>79.0–87.2</td>
</tr>
<tr>
<td>Epidemic (0.149)</td>
<td>20</td>
<td>18</td>
<td>78</td>
<td>55.9–90.2</td>
<td>68.3–86.6</td>
</tr>
<tr>
<td>Endemic (0.208)</td>
<td>21</td>
<td>36</td>
<td>85</td>
<td>63.1–94.8</td>
<td>81.2–90.0</td>
</tr>
</tbody>
</table>

**a** By TG-ROC analysis, cut-offs were determined for the p38-ELISA that optimally discriminate between infected aborting and other dams at risk using sera from herds with *N. caninum*-associated epidemic and endemic abortions.

**b** Analysis was done on epidemic and endemic herds (All), or restricted either to epidemic or endemic herds.

**c** Number of sera with positive (Pos.) or negative (Neg.) ELISA or 'gold standard' result.

**d** Equal sensitivity and specificity of the ELISA at the selected cut-off.

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