Use of an immunodominant p17 antigenic fraction of *Neospora caninum* in detection of antibody response in cattle

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A Neospora caninum *17 kDa protein fraction (p17) has been described as an immunodominant antigen (IDA) under reducing and non-reducing conditions. The aim of the present study was to investigate the diagnostic utility of p17 in cattle. In order to achieve this, p17 was purified by electroelution from whole* N. caninum *tachyzoite soluble extract and a p17-based Western blot (WB-p17) was developed. The p17 recognition was measured by densitometry and expressed as OD values to check the validity of the WB-p17. A total of 131 sera including sequential samples from naturally- and experimentally-infected calves and breeding cattle were analysed by WB-p17 and compared with IFAT using whole formalin-fixed tachyzoites as a reference test. The results obtained highlight the feasibility of using the* N. caninum *p17 in a diagnostic test in cattle. Firstly, the assay based on the p-17 antigen discriminated between known positive and negative sera from different cattle populations, breeding cattle and calves. Secondly, the p17 antigen detected fluctuations in the antibody levels and seroconversion in naturally- and experimentally-infected cattle. Significant differences in p-17 antigen recognition were observed between naturally infected aborting and non-aborting cattle, as well as significant antibody fluctuations over time in experimentally infected cattle, which varied between groups. Furthermore, the results obtained with WB-p17 are in accordance with the results obtained with the IFAT, as high agreement values were obtained when all bovine subpopulations were included ($\kappa = 0.86$).*

Key words: antibody response - *Neospora caninum* - cattle - Western bolt-p17

Neospora caninum, a cyst-forming coccidian parasite, has been identified as an important cause of abortion and neonatal mortality in cattle worldwide (Dubey 1999). Infection of animals with *N. caninum* elicits the development of characteristic immunoglobulin G (IgG) antibody responses against low molecular weight tachyzoite antigens (Bjerkas et al. 1994).

Preliminary work has suggested the 17 kDa immunodominant antigent as a possible antigenic marker since changes in its recognition could be related with acute infection or reactivation of a chronic infection (Alvarez-Garcia et al. 2002). The recognition of the p17 antigenic fraction is very specific for *N. caninum* infection and rabbit hyperimmune serum against *Toxoplasma gondii* did not react with this protein (Louie et al. 1997).

Therefore, the p17 antigenic fraction (p17) was selected to evaluate its potential as a diagnostic tool. To achieve this, a Western blot (WB) based on this antigen (WB-p17) was developed for the detection and quantification of serum IgG antibodies from naturally-and experimentally-infected cattle. All samples were also analysed by IFAT, which was regarded as the reference technique.

MATERIALS AND METHODS

Sera - Sera were collected from cattle naturally- and experimentally-infected with *N. caninum.* Serum samples from naturally-infected bovine animals were analysed and divided into three groups based on clinical findings and serological profiles – infected aborting and non-aborting breeding cattle as well as non-infected breeding cattle – as previously described (Alvarez-Garcia et al. 2003) (Table I). Cattle came from herds with a previous history of *Neospora* abortion problems in the last three years and proved to be seropositive by IFAT test.

Two sequential samples from six aborting breeding cattle were collected, one sample was recovered within a month of abortion, and the other one six months after or prior to the abortion, whereas a unique serum sample from infected breeding cattle along gestation (n = 6) and non-infected non-aborting breeding cattle (n = 6) were analysed.

In addition, precolostral sera from calves born to *N. caninum* infected (n = 4) and non-infected dams (n = 4) were also analysed. Samples were deemed to be seropositive if they had IFAT titers equal to or greater than 1:200.

Sera from experimentally-infected animals were obtained from the Moredun Research Institute (Edinburgh, Scotland). Animals had been infected with NC-1 isolate as previously explained (Innes et al. 2001). Briefly, the experiment was comprised of four groups of breeding cattle

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and three groups of calves born from breeding cattle groups II, III, and IV. Animals in groups III (infected with *N. caninum* prior to mating and challenged with *N. caninum* at mid-gestation) and IV (infected with *N. caninum* prior to mating and left unchallenged throughout pregnancy), had been inoculated with 10^7 *N. caninum* tachyzoites in a previous pregnancy and were inoculated with 10^8 live *N. caninum* tachyzoites, subcutaneously over the left prefemoral lymph node six weeks prior to mating. Cattle in groups II (challenged with *N. caninum* at mid-gestation) and III were each challenged at day 140 of gestation with 5×10^8 live *N. caninum* tachyzoites subcutaneously over the right pre-femoral lymph node. A control inoculum (5×10^6 Vero cells) was used to challenge cows in group I (uninfected controls). A summary of the samples analysed in the present work is shown in the Table II. Breeding cattle sera were obtained at four different times after challenge and for calves, two sequential serum samples were tested, precolostral and at day 5 after birth. All serum samples in this study were aliquoted and stored at –80°C prior to testing for specific antibodies by IFAT and WB-p17.

Parasites - In vitro maintenance of NC-1 isolate of *N. caninum*, purification of tachyzoites for IFAT and soluble antigenic extract were done according previously described procedures (Alvarez-Garcia et al. 2002).

Preparation and purification of p17 antigenic fraction - Whole soluble extract was obtained from tachyzoites (Alvarez-Garcia et al. 2003) and subjected to SDS-PAGE. Proteins were localized in gels by their molecular weight. Then the gel containing the protein of interest was cut into pieces and eluted by electrophoresis for 6-7 h at 10 mA/tube according to the manufacturer's instructions (Electro-Eluter Mod. 422, Bio-Rad Laboratories S.A., Alcobendas, Spain). After elution, the degree of purification was assessed simultaneously by SDS-PAGE combined with silver staining and Western blot using a *N. caninum*-positive bovine serum with an IFAT titer of 1:3200. Finally, proteins were quantified by comparison with standard concentrations of bovine serum albumin (BSA, Roche Molecular, Biochemical, Mannheim, Germany) in silver-stained SDS-PAGE gels.

IFAT test - Sera were analysed by IFAT in double serial dilutions starting at 1:50 using the previously described method (Trees et al. 1993). An IFAT titer of 1:200 was employed as a cut-off point.

WB-p17 - WB based on the purified p-17 fraction was carried out according to previously described procedures (Alvarez-Garcia et al. 2003). Equal amounts of this protein fraction were exposed to each bovine sample, which were employed at 1:25 dilution. The p17 recognition by the different sera samples was quantified using densitometry (Fluor-S™ Multimager, Bio-Rad Laboratories) and expressed as OD values. Quantity One® quantitation software (Bio-Rad Laboratories) was employed for the image analysis.

Statistical analysis - Differences in IFAT titers and OD values among the different groups of naturally-and experimentally-infected cattle studied (inter-group variations) were analysed by non-parametric Mann-Whitney *U*-test and Kruskal-Wallis H-test employing the STATVIEW package v 4.0 (Abacus Concept Inc., Berkeley, CA, US).

TABLE I

Naturally-infected cattle: groups and samples analysed

Breeding cattle				Precolostral calves	
Infected			Non-infected	Infected	Non-infected
Aborting cattle *a*	Aborting cattle *b*	Non-aborting cattle			
6	6	6	6	4	4

a: within a month of abortion; *b*: 6 months prior to or after abortion.

TABLE II

Experimentally-infected cattle: groups and samples analysed

	Breeding cattle					Calves		
Group	Week 0	Week 1	Week 4	Week 20	Total	Precolostral	Colostral	Total
I	5	5	5	5	20	4	4	8
II	6	5	6	6	23	6	6	12
III	6	6	6	6	24	6	6	12
IV	3	3	3	3	12	ND	ND	ND
Total	20	19	20	20	79	16	16	32

Group I: uninfected controls; group II: challenged with *Nespora caninum* at mid-gestation; group III: infected with *N. caninum* prior to mating and challenged with *N. caninum* at mid-gestation; group IV: infected with *N. caninum* prior to mating and left unchallenged throughout pregnancy; weeks after challenge at day 140 of gestation; ND: not done

To evaluate the effect of both variables – time and group – on antibody levels expressed as OD values, a parametric bifactorial Anova test was applied to the repetitive measures of antibody levels along time for all experimentally infected groups included in the study, and statistical calculations were computed using SAS v8.2. package (SAS Institute, Cary, NC, US). In this way, three null hypothesis (H_o) were defined: H_o^1: The variable "group" has no effect on antibody levels; H_o^2: There are no antibody fluctuations over time; H_o^3: All groups of cattle have similar antibody fluctuations over time.

The degree of agreement between IFAT and WB-p17 was estimated by calculating the kappa value (Thrusfield 1995).

RESULTS

Antibody profiles in naturally-infected cattle - Specific antibodies were detected by both serological tests in all samples from aborting and non-aborting infected breeding cattle (Fig. 1), whereas no positive results were obtained in negative controls. Although higher OD values and higher IFAT titers were found in samples collected in the month of abortion than in samples collected six months before or after the abortion in breeding cattle, there was no statistically significant difference between both samplings.

When differences between aborting and non-aborting dams were investigated, the intensity of recognition detected in the WB-p17 was higher in naturally-infected breeding cattle in the month of abortion (n = 6) compared with non-aborting breeding cattle (n = 6) ($P < 0.05$, Mann-Whitney *U*-test). Using IFAT, these differences were not statistically significant ($P > 0.05$, Mann-Whitney *U*-test). When samples taken 6 months before or after the abortion were included in the comparison (n = 12) the differences observed between infected aborting and infected non-aborting cattle were not significant ($P > 0.05$, Mann-Whitney *U*-test). When pre-colostral samples were tested, both serological tests discriminated well between positive and negative samples since all congenitally infected calves proved to be seropositive by both tests (Fig. 1).

Antibody profiles in experimentally-infected cattle - In breeding cattle OD values were present at high levels in groups III and IV during the entire sampling period. On the contrary, in group II recognition of p17 was not detected at weeks 0 and 1 following challenge, whereas moderate OD values were observed from week 4 after challenge at day 140 of gestation, reaching a high level at week 20 near parturition (Figs 2-3). This situation was similar when IFAT results were analysed. The results obtained when the parametric bifactorial Anova test was employed for comparing antibody fluctuations detected in the three infected groups permitted to reject the three null hypotheses previously stated. The antibody responses clearly depended on the group considered ($P < 0.0001$), rejecting H_o^1. There were significant antibody fluctuations over time (rejecting H_o^2), which varied between groups (rejecting H_o^3). Antibody fluctuations were statistically significant between weeks 0 and 1 and weeks 4 and 20 ($P < 0.0001$, Anova test). Differences in OD values and IFAT titers were statistically significant for each week post-infection among the three groups of infected breeding cattle ($P < 0.0001$, Kruskal-Wallis *H* test), except for week 20 in IFAT test as all groups showed similar high antibody titers.

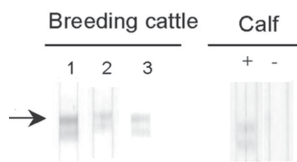

Fig. 1: recognition of the p-17 protein fraction by specific IgG antibodies in cattle naturally-infected with *Neospora caninum*. 1-2: aborting cow. 1: month of abortion; 2: six months before or after abortion; 3: infected breeding cattle. The recognition of the p-17 protein fraction is indicated with an arrow.

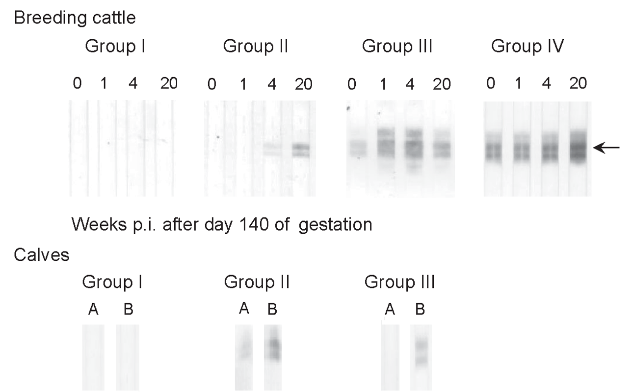

Fig. 2: recognition of the p-17 protein fraction by specific IgG antibodies in four groups of cattle experimentally-infected with *Neospora caninum*. A: precolostral sample; B: colostral sample. The recognition of the p-17 protein fraction is indicated with an arrow.

The WB-p17 discriminated well between positive and negative serum samples from calves born from experimentally-infected cows. Colostral antibodies were detected in groups II and III. Vertical transmission was only evident in calves from group II as antibodies against *N. caninum* were detected in precolostral sera by both tests (Fig. 3). Significant differences in antibody levels between two samplings in newborn calves were observed depending on the group and time of sampling ($P < 0.0001$, Anova test).

Agreement between IFAT and WB-p17 - The antibody profiles determined by IFAT were compared with those obtained with the purified p17 for naturally-and experimentally-infected cattle. High agreement values were obtained when all bovine subpopulations were included in the comparative study ($\kappa = 0.86$), as well as for groups of breeding cattle ($\kappa = 0.78$) and calves ($\kappa = 0.89$) when considered separately.

DISCUSSION

The immunodominant nature of the p17 suggests that this antigen could be an ideal candidate for the develop-

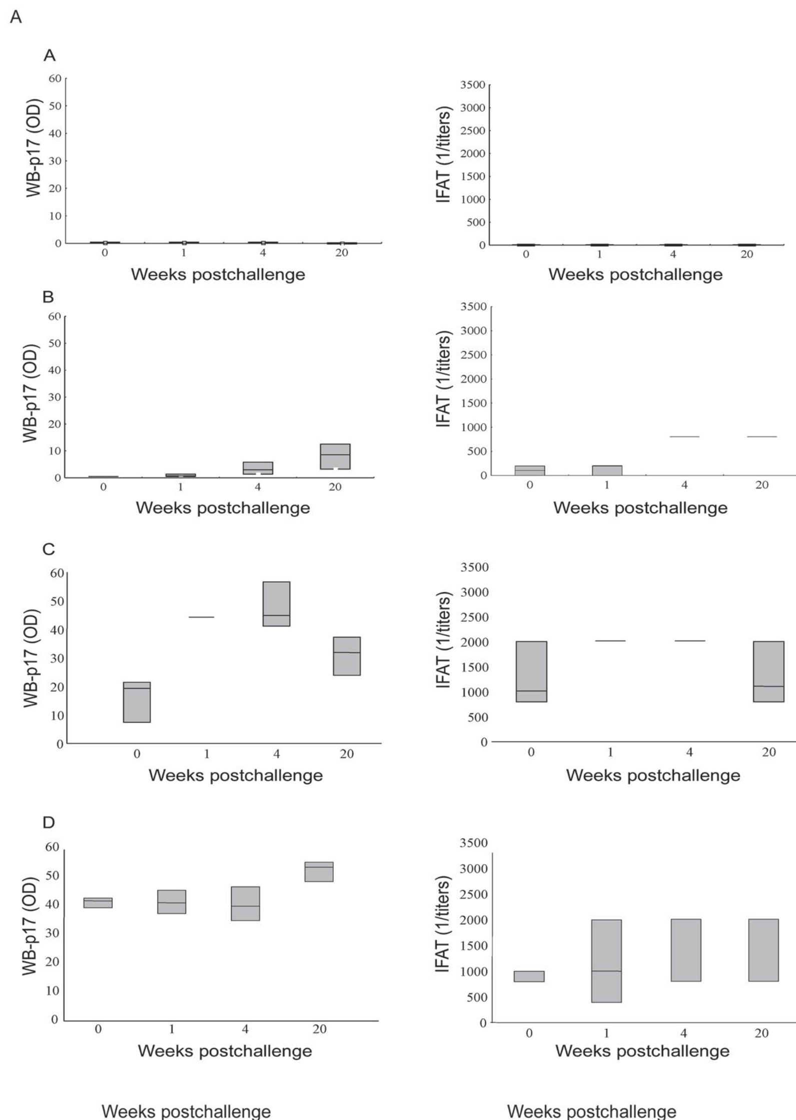

Fig. 3: box-plot graphs represent the lower, upper quartiles and median WB-p17 OD values and IFAT titers at different times after challenge at day 140 of gestation in four groups of cattle experimentally-infected with *Neospora caninum*. A: group I; B: group II; C: group III; D: group IV.

ment of serological diagnostic tests, since this antigenic fraction showed the highest frequency of recognition in naturally-infected breeding cattle and foetuses compared with other IDAs. The purified p17 was successfully employed in a quantitative diagnostic WB using densitometry to detect *N. caninum*- specific antibodies in sera. In our case, purified p17 was employed in a WB, following the procedure used in toxoplasmosis by Meek et al. (2003). Moreover, similar approaches have been previously reported for infectious diseases such as nocardiosis (Vera-Cabrera et al. 1992), where WB tests based on specific purified antigens were developed to investigate promising candidates for immunodiagnosis.

Several *N. caninum*-specific antigens have been employed in ELISA tests to diagnose *N. caninum* infection, proving to be highly sensitive and specific in the diagnosis of *N. caninum* infection in adult cattle. However, at present, the p-17 IDA has not been employed in the serodiagnosis of neosporosis.

The results obtained in the present study support the idea that this antigen could be a good candidate for the development of serological diagnostic tests as p-17 was detected in all infected cattle and differences in antibody levels among groups as well as fluctuating antibodies along time were detected by WB-p17 in a similar way as those use in other valuable tests. Furthermore, the requirements considered in the present study -the origin of the infection (naturally- or experimentally-infected cattle), the purpose of testing, sequential samples and different age groups- are desired when investigating the diagnos-

tic value of an antigen (Jacobson 1998, Greiner & Gardner 2000). As found in recent works (Gaturaga et al. 2005, Jenkins et al. 2005) sera from cattle infected by others biological related protozoa were not examined to test crossreactivity due to several reasons. Firstly, no cross-reaction has been observed between *Neospora* infection and *Toxoplasma, Cryptosporidium*, *Sarcocystis* spp., *Eimeria* spp. or *Babesia* spp. infection in cattle (Schares et al. 2000, Howe et al. 2002) when ELISA tests based on crude extract or recombinant proteins were developed to detect *Neospora* specific antibodies in cattle. Moreover, a few cross reactions between the most closely related species – *N. caninum* and *T. gondii* – have been detected in WB corresponding to a few number of antigens whose apparent molecular weight is higher than 29 kDa (Louie et al. 1997).

Initially, regarding the origin of the infection, p17 proved to have diagnostic value using sera from both natural and experimental infections. We found similar results using both techniques (IFAT and WB-p17) with samples from naturally-infected breeding cattle. In aborting breeding cattle higher IFAT titers as well as higher OD values were found in samples taken in the month of abortion, which is in accordance with the observations made by Quintanilla-Gozalo et al. (2000).

When differences between aborting and non-aborting naturally-infected breeding cattle were studied OD values were directly related with IFAT titers. Higher antibody levels were observed in aborting dams by both serological tests and our results agree with those obtained by others (Schares et al. 1998, Quintanilla-Gozalo et al. 2000). The statistically significant differences were only obtained with the WB-p17, so that according to our preliminary results future improvements of the assay based on p17 should involve optimisation of conditions, since one of the requirements of a serological test is to discriminate dams at risk of abortion from those infected dams without reproductive failure.

The results obtained for experimentally-infected breeding cattle are mainly in accordance with those reported in a previous work (Innes et al. 2001). The analysis of serial samples of breeding cattle throughout pregnancy permitted the detection of fluctuations in the IgG response directed against p17. In the animals in group II, which transmitted the infection to their descendents, antibody levels increased from week 4 after infection and remained lower than in the other breeding cattle groups mentioned above. The results from group III showed an increase of antibodies after the first week of reinfection, which remained high until parturition and in group IV, which did not transmit infection to the offspring, levels of antibodies remained high throughout the period of study until parturition. On the other hand, the differences observed in week 0 between groups III and IV may probably be due to individual differences since both groups are expected to present similar immune response. In general, fluctuations of antibodies detected by both tests in all groups are similar to those reported by others (Innes et al. 2001). The high recognition of p17 detected by sera from naturally- and experimentally-infected breeding cattle may be due to the fact that antibodies developed against p17 are a major specific IgG antibody fraction.

The p17 also permitted the detection of antibodies in precolostral sera which may be an important consideration in control programs of vertical transmission (Thurmond & Hietala 1995). Our results confirm the diagnostic value of p17, previously pointed out by other authors (Louie et al. 1997).

The potential value of p17 in serological diagnosis is also supported by the high agreement values obtained between IFAT and WB-p17 for the different groups included in the comparative study. Variable agreement results have been obtained even when comparing similar serological tests (von Blumroder et al. 2004).

The use of p17 in serological tests may offer advantages over whole tachyzoite soluble extract, since antibodies directed against this protein represent a major specific IgG fraction in infected animals. The reactivity of bovine infected sera against the reduced antigen was easily detectable, indicating that this highly immunogenic antigen could be an optimal marker to be employed in an ELISA test based on a recombinant antigen.

In future studies, an ELISA test utilizing this antigen alone or in combination with other specific antigens may help us to improve the sensitivity, specificity and scope over existing diagnostic tests.

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