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Short communication

Prevalence of *Neospora caninum* infection in Australian (NSW) dairy cattle estimated by a newly validated ELISA for milk

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Abstract

Aim: To determine the performance characteristics of an Institut Pourquier (IP) enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *Neospora caninum* in bovine milk and subsequent determination of the prevalence of *N. caninum* infection in New South Wales (NSW) dairy cattle.
Methods: Matching serum and milk samples from 93 cattle were assayed in two commercially available ELISAs for the detection of anti-*N. caninum* antibodies. Serum test results of one ELISA (IDEXX) were used to determine the *N. caninum* infection status of the cattle. Optimised cut-off values for the IP ELISA using milk samples were determined by two-graph receiver operating characteristic (TG-ROC) analysis and then applied to a representative sample of 398 milk samples from dairy herds around NSW.
Results: When this ELISA was applied to a representative collection of 398 milk samples from dairy cattle across NSW it demonstrated a 21.1% prevalence of *N. caninum* infection in those cattle. From the TG-ROC analysis an IP ELISA protocol was derived which suggested a cut-off threshold that would allow milk testing with 97% sensitivity and specificity, respectively, relative to serum testing.
Conclusions: The prevalence of *N. caninum* in NSW dairy cattle was higher than previously believed. When used on individual milk samples this ELISA demonstrated high sensitivity and specificity and so could be used to accurately identify *N. caninum* infection. TG-ROC analysis of the IP ELISA optimised the protocol and prescribed cut-off values enabling the ELISA to be used for the screening of *N. caninum* antibodies in the milk of dairy cattle.

Keywords: *Neospora caninum*; Protozoa; Cattle; Serology; Milk; ELISA; ROC analysis; Prevalence

1. Introduction

Neospora caninum is well known as a cause of abortion in beef and dairy cattle in many countries around the world, including New Zealand and Australia (Reichel, 2000) where it often causes abortions of epidemic (‘‘storm’’-like) proportions (Thornton et al., 1994; Atkinson et al., 2000). Diagnosis of *N. caninum* infection in these cases has been based largely on the serological testing by ELISA of serum from all or parts of the affected dairy herds. ELISA assays have also been described for use with milk samples (Björkman et al., 1997; Schares et al., 2004), however widespread testing for *N. caninum* antibodies in milk is not yet a common practice worldwide. Here we describe an adaptation of a serum-based ELISA for testing milk

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samples for the presence of antibodies to *N. caninum* and its subsequent use in a state-wide seroprevalence study of NSW dairy cattle.

In the present study, the performance of an indirect ELISA (Institut Pourquier) on milk samples was analysed, and the results compared with an indirect *N. caninum* ELISA (IDEXX) on corresponding sera. From this the performance characteristics of the milk assay, including cut-off thresholds and Se and Sp were derived. Test-and-cull programs for the control of *N. caninum* in cattle have previously been described (Hall et al., 2005) and this ELISA using milk may become a useful tool in such control programs.

2. Materials and methods

2.1. Sampling

The serum samples came from three dairy herds (Herds 1, 2 and 3) located in NSW, Australia. Herd 1 (Hall et al., 2005) and Herd 2 were the subject of *N. caninum* investigations that involved whole herd bleeds. Only a few samples were sourced from Herd 3. A total of 93 corresponding milk samples were collected from both seropositive and seronegative cows.

2.2. IDEXX ELISA

The serum samples were assayed in duplicate for anti-*Neospora* antibodies using a commercially available indirect ELISA (5N05.00) (IDEXX Laboratories, Westbrook, Maine, USA). The results of this ELISA were used as the ''reference standard'' to determine the *N. caninum* infection status of the cows. This ELISA is known to be an appropriate ''reference standard'' (von Blumröder et al., 2004).

2.3. Indirect Institut Pourquier (IP) ELISA

2.3.1. Determination of optimal dilution

A subset of 55 milks from cows with established *N. caninum* infection status (see Section 2.2) were assayed in the indirect IP ELISA (P00511/01) with slight modification. Milk samples were assayed undiluted (neat), 1/2 and 1/3 in dilution buffer supplied with the ELISA kit and the rest of the assay was run as suggested by the manufacturer. Positive and negative control sera delivered with the ELISA kit were used as controls in the milk ELISA. Data were analysed to determine the optimal dilution of milks for this ELISA.

2.3.2. Optimisation of cut-off values

The 93 milk samples from dairy cows for which the infection status had been determined in the IDEXX ELISA (see Section 2.2) were assayed in the IP indirect *N. caninum* ELISA. The milks were tested at the optimal dilution as determined in Section 2.3.1. Results were expressed as the ratio of the mean absorbance values of the Sample (S) to the mean absorbance value of the Positive (P) control sample provided with the diagnostic kit. The resultant S to P ratio was expressed as a percentage (S/P%). The manufacturer recommended that sera with S/P% $\leq$ 30% be regarded as negative, S/P% of between 30 and 50% regarded as suspicious and S/P% $\geq$ 50% regarded as positive sera. However, there was no specific cut-off threshold set for milk samples. A recent study has determined that a more appropriate cut-off for serum samples is 27 S/P% (Hall et al., 2006).

2.3.3. Analysis of serological data

The computer program CMDT was used for the computation of the performance characteristics of the IP *N. caninum* ELISA when applied to milk samples. Characteristics, such as Se, Sp and the optimal cut-off threshold were determined. Two-graph receiver operating characteristic analysis (Greiner, 1995) was used to assess the diagnostic performance of the ELISA. This calculates and plots the effects of varying cut-off values on both Se and Sp. A cut-off value was derived from the TG-ROC plot, using parametric and non-parametric methods, as the S/P%-value (d0) where Se and Sp were equal (theta0).

2.4. Milk prevalence survey

Milk samples from individual cows (n = 398) were obtained at random from 203 dairy farms across NSW. Two samples were collected from 195 properties and in another eight cases only one sample was tested as the other had curdled. The majority of the samples were collected by regular herd samplers (through Dairy Express) at the time of herd recording. The samples were collected with no preservative, stored at 4 °C overnight and then sent express to a laboratory where they were stored at −20 °C until tested. Samples were centrifuged at 1000 × g, skim milk collected and assayed as per the optimal ELISA conditions determined in Section 2.3.1, applying the cut-off value obtained in Section 2.3.2. The results were displayed as prevalence by shire. A map displaying the distribution of positive and negative farms was also