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Bulk-tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds

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Abstract

Paratuberculosis (Johne's disease) has been widespread in Danish dairy herds for a long time but the herd-level prevalence has never been determined precisely. To evaluate the prevalence of paratuberculosis in Danish dairy herds in various regions, an ELISA based on a commercially available antigen was adapted for testing bulk-tank milk for the presence of antibodies to *Mycobacterium avium* subsp. *paratuberculosis*. Bulk-tank milk samples were collected from six milk-collecting centres from six different areas of the country. Samples from 900 herds (about 7.5% of all Danish dairy herds) were examined, and 70% were positive at the statistically optimal cut-off (sensitivity 97.1%; specificity 83.3%). The technical performance of the ELISA was not sufficient to provide a tool for surveillance because even slight changes in optical density for the samples would change the classification of some samples. The infection is more widespread than previous investigations have shown.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Cattle-microbiological diseases; Johne's disease; ELISA; Bulk-tank milk

1. Introduction

Paratuberculosis (Johne's disease) is a chronic granulomatous enteritis in cattle; the agent is *Mycobacterium avium* subsp. *paratuberculosis* (Chiodini et al., 1984). The

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prevalence of infection in most countries in the world is unknown, but studies from some countries indicate a herd prevalence ranging from almost 0% in Norway and Sweden (Viske et al., 1997; Anon., 1998; Djønne et al., 1998) to 21% in the USA (NAHMS, 1997). Paratuberculosis in cattle has occurred in Denmark for almost a century, but reliable estimates of the prevalence of the disease are not available. In Jutland in 1961, 2.5% of blood samples from 1875 cattle >2 years old were positive in the complement-fixation test (CFT) (Ringdal, 1964); in Zealand, the sero-prevalence was 1.8%. Berg Jørgensen (1965) reported a prevalence of 2.3% in adult cattle and 0.45% in cattle <2 years old. Flensburg and Munck (1980) estimated a herd-level prevalence of 5–6% on the island of Funen based on observations of clinical paratuberculosis and subsequent CFT on blood samples from all adult cattle present in the examined herds.

The purpose of this study was: (1) to adapt an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *Mycobacterium avium* subsp. *paratuberculosis* in bulk-tank milk and (2) to estimate the herd-level prevalence of paratuberculosis in Danish dairy herds based on use of this ELISA.

2. Material and methods

2.1. Selection of herds

In early 1998, 900 bulk-tank milk samples were collected from six milk-collecting centres from various parts of Denmark (Table 1, first two columns). The size and composition of the herds were as follows: minimum: 2 cows; 25th percentile: 31 cows; median: 48 cows; 75th percentile: 70 cows; maximum: 512 cows.

The milk samples from the various milk-collecting centres were collected from a geographically small area within the area of the milk-collecting centre.

Table 1
Herd-level prevalence of paratuberculosis estimated on the basis of results of ELISA testing on bulk-milk from selected Danish milk-collecting centres. The prevalence is given at three different cut-points, with 0.02 being the statistical optimal cut-point

Centre	Number of herds tested	Cut-off in ELISA test (OD_C)					
		0.01		0.02		0.05	
		Number positive	True prevalence (%)	Number positive	True prevalence (%)	Number positive	True prevalence (%)
Northern Jutland	100	58	78	43	57	29	38
Mid-Jutland	150	64	57	41	5	19	15
Southern Jutland	200	173	100	131	89	39	24
Funen	200	145	99	98	44	30	18
Zealand	200	190	100	181	100	130	88
Bornholm	50	11	28	3	5	0	0

Samples were collected for testing from the Danish milk-quality program, where samples are collected once a week without prior notice to the farmer on certain days chosen by the milk-collecting centre; hence, the samples used in the current examination were collected using convenience sampling. The total number of Danish dairy herds was approximately 12 000 at the time of sampling.

In addition, 45 bulk-milk samples were collected from Danish herds where individual cows infected with *M. avium* subsp. *paratuberculosis* had been cultured from faeces. At least one positive cow was detected in each of these herds, but the within-herd prevalence was different from one herd to another. These 45 samples were used as positive reference material. Ninety-six bulk-tank milk samples from Norway were used as negative reference material; Norway was considered free of bovine paratuberculosis at the time of sampling (Mørk and Sund, 1997).

After receiving the milk samples, the lipid fraction was removed, and the skimmed milk was frozen at −18°C until testing. No chemical preservation of the milk samples was used.

2.2. ELISA

An ELISA based on a commercially available antigen[1] was adapted in co-operation with Klausen from the Danish Veterinary Laboratory in Copenhagen. The antigen was *M. paratuberculosis* Strain 18, actually an *M. avium* subsp. *avium* serotype 2 (Chiodini, 1993). The test was originally described by Abbas et al. (1983) and later modified by Vannuffel et al. (1994) and Hardin and Thorne (1996).

Microtiterplates (Polysorp®[2]) were coated with antigen (1.25 μg/ml in 0.1 M carbonate-buffer, pH 9.6) and the coated plates were left overnight at 5°C. Milk samples to be tested were incubated overnight at 5°C after addition of an equal amount of a suspension of *Mycobacterium phlei*-sonicate[3] (4 mg/ml). The coated plates were washed four times in phosphate-buffered saline (pH 7.4) with 0.05% $Tween_{20}$ and the treated samples were added to the wells in duplicate (100 μl/well) without further dilution and left for overnight incubation at 5°C. On each plate, two sets of positive and negative controls were applied. After repeated washings, 100 μl of a 1:500 dilution of goat anti-bovine IgG (H+L) conjugated to peroxidase[4] was added to each well. Plates were incubated for 1 h at 20°C and washed again. *Ortho*-phenylene-diamine (OPD)[5] was dissolved in citrate-buffer (pH 5.0) to a concentration of 300 μg/ml and supplemented with 80 μl H_2O_2, per 100 ml of the substrate solution, after which 100 μl was added to each well. Finally, 100 μl 0.5 M H_2SO_4 per well was used to stop reactions and the absorbence was read in an ELISA reader at 492/620 nm.

The reactions were stopped before the negative control reached an OD of 0.100.

[1] Allied Monitors, Fayette, Missouri, USA.
[2] Nunc, Roskilde, Denmark.
[3] Kindly provided by J. Klausen, Danish Veterinary Laboratory, Copenhagen, Denmark.
[4] Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA.
[5] Kem-En-Tec, Copenhagen, Denmark.

3. Statistical analyses

To compensate for non-specific reactions, a corrected optical density (OD_C) was calculated for each sample by subtracting the OD of the negative control of each plate from the OD of the test sample.

OD_C-values for the positive and negative reference materials were used for calculation of test sensitivity and specificity. A receiver-operating characteristic (ROC) curve was created based on the calculated sensitivity and specificity at various cut-off values (Hanley and McNeil, 1982; Jensen and Poulsen, 1992). The statistically optimal cut-off value of the test was determined using differential positive rate (DPR) curves. DPR is defined as: test sensitivity ÷ (1 ÷ test specificity) (Jensen and Poulsen, 1992).

The true prevalence was calculated as defined by Martin et al. (1987)

$$p(D+) = \frac{p(T+) - (1 - \mathrm{Sp})}{1 - ((1 - \mathrm{Sp}) + (1 - \mathrm{Se}))}$$

where $p(D+)$ is the true prevalence, $p(T+)$ the apparent prevalence and Se and Sp are the sensitivity and specificity of the applied test.

4. Results

The sensitivities and specificities calculated from the results obtained for the positive and negative reference material at various cut-off values are shown in Table 2. Values

Table 2
Sensitivities and specificities calculated at various cut-off-values with 95% confidence limits. True positive and true negative is defined as infected herds, not diseased herds

Cut-off (OD_C)	Sensitivity[a]		Specificity[b]	
	%	95% CI	%	95% CI
0.00	100	87.100	29	21.40
0.01	97	82.100	67	56.76
0.02	97	83.100	83	74.90
0.03	85	68.95	90	81.95
0.04	82	65.93	95	88.98
0.05	74	55.87	97	91.99
0.06	71	52.84	99	94.100
0.07	62	44.77	99	94.100
0.08	56	38.72	100	95.100
0.09	56	38.72	100	95.100
0.10	53	35.70	100	95.100
0.11	50	33.67	100	95.100
0.12	47	30.65	100	95.100
0.13	44	28.62	100	95.100
0.14	44	28.62	100	95.100
0.15	41	25.59	100	95.100

[a] n=45 Danish herds with positive faecal cultures for *Mycobacterium avium* subsp. *paratuberculosis*.

[b] n=96 Norwegian herds presumed to be free of paratuberculosis because Norway was considered infection-free at the time of sampling.

were entered in a chart and the peak of the curve (OD_C=0.020) was identified as the statistically optimal cut-off value (sensitivity 97.1% and specificity 83.3%). This cut-off value for the 900 test samples gave a distribution of positive and negative herds (Table 1).

Table 1 also shows the distribution if the cut-off value is reduced to 0.010 or increased to 0.050, respectively.The ROC-curve had area under the curve calculated to be 0.96 (on a 0–1 scale).

5. Discussion

The antigen used in the present test was prepared from *M. avium* subsp. *avium* and not from *M. avium* subsp. *paratuberculosis*. An antigen specific to paratuberculosis might be advantageous, but neither a study of the literature nor results from and comparison of a subsp. avium ELISA with a subsp. paratuberculosis ELISA in our laboratory (data not shown) points to the existence of such an antigen. The most-immunogenic regions of the genome of the two subspecies appear to be identical (de Kesel et al., 1992; Coetsier et al., 1998). An antigen (named ''a362'') from the most-immunogenic regions of the genome tended to be specific of *M. avium* subsp. *paratuberculosis* (de Kesel et al., 1993; Vannuffel et al., 1994), but the material presented was based on too few samples to be definitive. Rather, de Kesel et al. (1993) demonstrate merely a close immungenic relationship between the two mycobacteria.

We assumed that Norway was free of paratuberculosis, but after the start of this study, reports have indicated that Norway may not be totally free of paratuberculosis in cattle (Anon., 1998) and at least four infected herds have been identified.[6] However, the Norwegian samples generally gave very low OD-values as compared to the Danish samples. Thus, the assumption that Norway has a relatively low prevalence of paratuberculosis seems to be justified from the samples used in this study. It was also assumed, that a possible exposure to avian tuberculosis in the examined herds was the same in the Danish herds as in the Norwegian herds. The area under the ROC curve of 0.96 indicated both a high sensitivity and a high specificity even though the test antigen is from subspecies avium. This supports the assumption that the level of exposure to avian tuberculosis is similar in the herd material from the two countries.

In this report, 70% (35, 97%) of the examined herds were positive in the ELISA test (OD_C=0.02). Hence, paratuberculosis is more widespread in Danish dairy herds than previously reported but we do not know the cow-level prevalence of paratuberculosis in these herds, and the level of infection necessary to detect the disease. Flensburg and Munck (1980) reported much lower prevalence, but their estimate was based on voluntary registrations. The present study was an anonymous study; thus, no farms were omitted due to fear of registrations.

[6] Djønne, B., Holstad, G., Kolbjørnsen, Ø., Nyberg, O., Schönheit, J., Tharaldsen, J., Ødegaard, Ø., 1999. Is paratuberculosis in goats a source of infection to cattle? Some preliminary results from a National Surveillance Program in Norway. Poster abstract presented at the Sixth International Colloquium on Paratuberculosis, 14–18 February 1999.

Two recent Dutch studies have indicated a prevalence of 50–70% among the dairy herds in the northern provinces of The Netherlands.[7,8] The situation appears to be identical in Danish dairy herds. To further investigate this and to find samples that would give a more-specific diagnosis, faecal culturing could be used. This method is highly specific, but the sensitivity is low. In one study the sensitivity was estimated to 55% (Sockett et al., 1992), but the material was based on faecal shedders only not on all infected animals. Rather, data from our laboratory suggest a sensitivity of the faecal culture method to be 20–30% (data not shown), but sampling of these data were also biased. Because the faecal culture method has such low sensitivity, it has little value for screening purposes, unless all cattles are tested.

The ELISA test studied here has limitations. Small variations in the cut-off value will affect the estimated prevalence dramatically (Table 1), so the test is not sufficiently reliable. Major technical improvements in the assay are still needed.

6. Conclusions

The overall herd-level prevalence of paratuberculosis in the dairy herds investigated was approximately 70% which means that eradication may be very difficult. The ELISA described here can be used with some modifications for detection of high-prevalence herds but not herds with a very low prevalence; and cow-level testing will be needed there.

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