Use of a dot enzyme-linked immunosorbent assay on absorbed sera for the diagnosis of bovine paratuberculosis


Summary: This study describes the response of cattle to a dot enzyme-linked immunosorbent assay (ELISA) using sera absorbed with Mycobacterium phlei. Results obtained by visual observation are compared with those obtained using a densitometer. Infection status of cattle was determined by faecal culture. Cattle of different levels of exposure and disease manifestation were examined.

A significantly higher dot ELISA response was observed (using both absorbed and non-absorbed sera) in animals with heavy shedding of M. paratuberculosis than in animals which tested negative by faecal culture or shed M. paratuberculosis at lower levels (P < 0.05).

Paratuberculosis was diagnosed by visual determination of dot ELISA results using non-absorbed sera in 29 of 44 (65.9%) clinically-suspect animals giving positive results by faecal culture, and 85 of 93 (91.4%) cattle testing negative by faecal culture. With absorbed sera, the sensitivity of visual determination decreased to 15 of 44 (34.1%), while specificity increased to 91 of 93 (97.8%). Approximately 75% of cattle yielding positive results by dot ELISA were heavy bacterial shedders (> 1,500 colonies/g of faeces) at the time of serological testing.

Comparison of the dot ELISA results determined visually with results obtained by objective densitometric measurement showed compatible specificity. Sensitivity of the dot ELISA was 65.9% for non-absorbed sera using visual evaluation and 87.5% using densitometric evaluation at a cut-off optical density value of 0.2. For absorbed sera, the values were 34.1% and 82.5%, respectively.


INTRODUCTION

Serological testing for paratuberculosis has often suffered from a lack of diagnostic accuracy (4). An attempt to increase diagnostic specificity using test sera absorbed with Mycobacterium phlei in an enzyme-linked immunosorbent assay (ELISA) has been
reported by Yokomizo and colleagues (15). Briefly, 50 µl of test sera was mixed with 950 µl of heat-killed salt suspension (5 mg [dry weight]/ml) of \textit{M. phlei} and allowed to react at room temperature for 1 h. The mixture was then centrifuged at 1,200 g for 30 min at 4°C.

Milner and colleagues (8) modified the above serum absorption technique by adding 40 mg of \textit{M. phlei} to 1 ml of a 1:100 dilution of serum in ELISA buffer, which was then left overnight at 4°C. Immediately before use, the \textit{M. phlei} was removed by centrifugation at 12,000 g for 5 min. Both techniques were investigated in pilot experiments at the National Veterinary Laboratory (NVL) in Copenhagen (Denmark), and results were found to be compatible. The method described by Milner was chosen, as the overnight processing facilitated the planning of workloads in the laboratory from day to day. The only modification introduced was that instead of using live \textit{M. phlei} for absorption, organisms were killed by autoclavation for 15 min at 121°C and a pressure of 103.5 kPa (15 lb/in²). In other pilot studies, no difference was reported in results using killed \textit{M. phlei} in this ELISA method (unpublished findings).

The purpose of this study was to describe the response of three different groups of cattle to a dot ELISA test for paratuberculosis, using sera absorbed with \textit{M. phlei}, as determined visually and also by densitometric measurement using a computer-vision software system.

**MATERIALS AND METHODS**

**Study herds**

Sera from three groups of cattle were tested:

- Group 1 contained cattle (n = 52) from two herds giving negative results by faecal culture
- Group 2 contained suspect cases (n = 40) of paratuberculosis confirmed by faecal culture
- Group 3 contained cattle (n = 45) from two herds known to be subclinically-infected with paratuberculosis (diagnosed on the farm).

All of the cattle used in the study belonged to three breeds: Danish Holstein, Red Danish Milkrace and Jersey. All animals older than twelve months were tested twice with a six-month interval. The two herds in group 1 had no history of paratuberculosis. Group 2 was constituted on the basis of bovine faecal samples submitted by various owners to the NVL. The herds from which cattle in group 3 were taken had no clinical cases of paratuberculosis during the twelve-month study period.

**Faecal and serum samples**

Samples were collected from the above herds during a previous study (2), with the exception of the forty samples from group 2, which were obtained just prior to this study. Faecal samples were taken from the rectum and blood was drawn from the jugular vein. Processing of the material in the laboratory commenced 18-24 h after sampling and faecal cultures were performed 24-48 h after sampling.

Sera were separated by centrifugation and aspiration, and stored at −70°C for subsequent testing.
Absorption treatment

*M. phlei* (40 mg wet weight) was added to 1 ml of a 1:100 dilution of serum in ELISA buffer (see “Dot ELISA serology” below), and stored at 4°C overnight. Immediately before use, *M. phlei* was removed by centrifugation at 12,000 g for 5 min.

Faecal culture

Faecal samples were cultured as previously described (1). Samples of faeces (3 g) were suspended in a 4% solution of sodium hydroxide and a 5% solution of oxalic acid containing 0.1% malachite green. After centrifugation, the sediment was suspended in 4 ml of 0.15 M saline and 0.1 ml of the suspension was inoculated into each of four tubes with Löwenstein-Jensen medium containing 0.16 g mycobactin P, 4 g sodium pyruvate, 0.75 g cycloheximide, 0.2 g chloramphenicol and 200,000 units of benzylpenicillin sodium (1).

Colonies of bacteria were counted after incubation for twelve weeks at 37°C. Non-chromogenic, slowly-growing colonies were classified as *M. paratuberculosis* (1) and subcultures were made on Löwenstein-Jensen media with and without mycobactin P. Mycobactin dependence and colony morphology were the diagnostic criteria for *M. paratuberculosis*. Results of faecal culture were expressed as the number of viable *M. paratuberculosis* colonies per g of faeces processed. The following categories were distinguished:

- +: 1-150 colonies/g
- ++: 151-700 colonies/g
- +++: 701-1,500 colonies/g
- ++++: > 1,500 colonies/g.

Antigens used in ELISA

Sonicates of *M. paratuberculosis* (ATCC strain 19698) were used as antigens. A wet weight of 200 mg of the organism was suspended in 20 ml phosphate-buffered saline (PBS) pH 7.2, and sonicated on ice at 4°C for two periods of 15 min each, then centrifuged at 10,000 g for 30 min at 4°C. The supernatant was harvested (4 mg per ml of protein), aliquoted and stored at −70°C until use. *M. phlei* (strains 1 to 4) routinely used for mycobactin production at the NVL was used to absorb serum.

Dot ELISA serology

The method described by Woodruff and colleagues (13) was used, with several modifications, to detect antibodies to *M. paratuberculosis*. Nitrocellulose sheets (pore size 0.45 µm) were saturated with tris-buffered saline solution (TBSS; 20 mM tris, 500 mM NaCl, pH 8.2) and placed in a 96-well acrylic manifold. Using forceps and gloves, the nitrocellulose sheet was placed across the surface of the filter support. The sample cell was placed on top of the nitrocellulose filter and the manifold was tightened. Excess TBSS was removed by vacuum, and 100 µl of antigen in a dilution of 1:100 (0.4 µg of protein) was introduced by pipette into 48 of the 96 wells, in alternating rows. The remaining rows of wells received 100 µl of TBSS only and were used to evaluate non-specific binding of antibody. The loaded manifold was then incubated for 15 min at 37°C. The antigen and TBSS solutions were removed by vacuum and 300 µl of TBSS containing 2% (v/v) Tween 20 was introduced by pipette into each of the 96 wells to block unbound sites on the nitrocellulose (3). The manifold was incubated for 5 min at 37°C, and the blocking solution removed by vacuum. The wells were then washed three
times for 2 min each by pipette using 200 µl of a TBSS washing solution (55 mM tris, 150 mM NaCl, 0.025% Tween 20, pH 8.2). Test sera were diluted 1:1,600 using TBSS containing 0.5% (v/v) Tween 20 as the diluent, and 100 µl were applied to each well. Optimum serum dilution was determined by checkerboard titration using sera from animals testing positive and negative by faecal culture, as well as control sera positive for salmonellosis and bovine leukaemia virus infection. Each test serum solution was introduced by pipette into two wells containing antigen and two containing no mycobacterial antigen. The test sera were incubated for 15 min at 37°C, and following evacuation each well was washed three times as above. Subsequently, 100 µl of a 1:4,000 dilution of horseradish peroxidase-conjugated rabbit anti-bovine immunoglobulin (Ig)G (heavy and light chains) in TBSS containing 0.5% Tween 20 was added to each well. The conjugate was incubated for 15 min at 37°C. The wells were again washed three times and the nitrocellulose paper was removed from the acrylic manifold. The reaction was blocked before development in 2% Tween 20 in TBSS for 5 min and washed three times. The wells were then placed in a sodium acetate buffer (50 mM, pH 5.0) for 10 min before being placed in a freshly prepared substrate solution (12.5 ml of 0.4% dioctyl sodium sulfasuccinate [DOSS] solution in 96% ethanol, 0.5 ml tetramethylbenzidine [TMB] 5.0% solution in acetone, 50 ml sodium acetate buffer [50 mM, pH 5.0] and 25 µl of 30% hydrogen peroxide) for about 2 min. The colour development reaction was stopped by transferring the membrane to double-distilled water for 5-10 min, and then to a post-development solution of 13 ml DOSS and 50 ml distilled water for an additional 10 min. The membrane was allowed to air dry before reading and then stored between two pieces of filter paper, wrapped in tin foil and refrigerated.

Positive and negative reference sera were assayed on each membrane. Each sample was run in duplicate on each membrane, and each serum from the same animal was used non-absorbed as well as absorbed with *M. phlei*. Results were determined by reading the membrane by video densitometric measurement using a computer-based system. Briefly, the video images were divided into small squares and digitalized in values between 0 and 250; the numbers (referred to as pixel values) are proportional to the light intensity of the image. All pixel values of the dot blots were integrated and the value of the background reading was subtracted. Results were expressed as a percentage of the positive reference serum (6).

For visual reading, a positive test result was recorded if any blue colour was detected in the test dot. A negative test result was recorded if no colour appeared or if the colour of the control dot containing no antigen was nearly as intense as the colour of the test dot (13).

**Data analysis**

Using densitometric measurements, the end result of an ELISA test performed with a single serum dilution was expressed in quantitative optical density (OD) units, rather than as a qualitative (positive/negative) result obtained visually. If there is some overlap between ELISA results for infected and uninfected cattle, as is the case in many ELISA tests for paratuberculosis, some information is lost when results are expressed as simply positive or negative. Sensitivity (defined as the rate of positive ELISA results for faecal culture-positive cattle) and specificity (defined as the rate of negative ELISA results for faecal culture-negative cattle) of the test were calculated for different cut-off levels.

The positive predictive value was defined as the rate of true-positive ELISA results (ELISA-positive and faecal culture-positive) expressed as a percentage of all ELISA-
positive results, and the negative predictive value was defined as the rate of true-negative ELISA results (ELISA negative and faecal culture-negative) as a percentage of all ELISA-negative results. The T test was used for comparison of means. A probability value of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

Results of dot ELISA testing of the samples from the three groups of study herds were determined by visual reading as well as by an objective measure (see “Materials and methods”).

Visual reading was performed on serum samples from all three groups. Twenty-nine of the 44 (65.9%) cattle testing positive by faecal culture (40 from group 2 and 4 from group 3) and 85 of the 93 (91.4%) faecal culture-negative cattle (52 from group 1 and 41 from group 3) were correctly identified using the visual reading of dot ELISA when testing non-absorbed sera. Absorbed sera from 15 of 44 (34.1%) faecal culture-positive cattle gave positive results, while 91 of 93 (97.8%) faecal culture-negative cattle tested negative using absorbed sera.

Of the 29 faecal culture-positive cattle which tested positive by dot ELISA using non-absorbed sera, 22 (75.9%) were shedding pathogen at a rate of more than 1,500 colonies/g of faeces at the time of serological testing. Similarly, of the 15 faecal culture-positive cattle which tested positive using absorbed sera, 11 (73.3%) were shedding pathogen at a rate of more than 1,500 colonies/g of faeces at the time of serological testing.

**Mean dot ELISA values of the three groups of study animals**

The mean dot ELISA values (percentage of the positive reference serum; $P < 0.05$) of group 1 were 37.9% using non-absorbed sera and 25.0% using absorbed sera.

The results for group 2 were analyzed in relation to the number of colonies isolated from the animals (see “Materials and methods”), and thereby in relation to the amount of shedding in each animal when the faecal sample was taken for culture. The mean dot ELISA value was 55.0% for + shedders, 53.0% for ++ shedders, 47.0% for +++ shedders and 71.3% for ++++ shedders using non-absorbed sera, and 49.0%, 47.0%, 53.0% and 65.8% of positive reference sera when using the same sera absorbed with *M. phlei*. For both absorbed and non-absorbed sera, a statistically significant difference was found between low (< ++++) and high (> ++++) *M. paratuberculosis* shedders ($P < 0.05$).

The two herds in group 3 each contained two faecal culture-positive individuals: one herd contained a + shedder and a ++ shedder, while the other herd contained two + shedders. The mean dot ELISA value for group 3 was 47.3% using non-absorbed sera and 48.6% using absorbed sera ($n = 41$).

The frequency distribution of faecal culture-negative and -positive cattle demonstrates a considerable overlap in the distribution of dot ELISA response. For this reason, selection of the optimum cut-off point was not intuitively obvious, since the sera could not always be clearly segregated into two groups. The cut-off value which provides maximum diagnostic sensitivity and specificity can be calculated by adding together the sensitivity and specificity for each chosen cut-off value. This allows the use of more than one cut-off value, and enables interpretation of calculated sensitivity and specificity for a wide range of cut-off values.
Sensitivity and specificity of the dot ELISA using both absorbed and non-absorbed sera were calculated for various cut-off levels (Table I). The sensitivity of the dot ELISA in group 2 was 92.5% with a cut-off optical density (OD) value of < 0.1, and 82.5% at cut-off OD values of 0.1 and 0.2 (Table I). The corresponding positive predictive values were 100%, as every animal in group 2 had tested positive by faecal culture.

### Table I

**Effect of absorption of sera with Mycobacterium phlei on dot enzyme-linked immunosorbent assay (ELISA) for Mycobacterium paratuberculosis in three groups of cattle**

<table>
<thead>
<tr>
<th>Faecal culture results for each group (a)</th>
<th>Dot ELISA using absorbed serum</th>
<th>Dot ELISA using non-absorbed serum</th>
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**Notes:**
- a) group 1: faecal culture-negative cattle; group 2: faecal culture-positive, clinically-suspect cattle; group 3: two subclinically-infected herds
- b) mean value expressed as a percentage of optical density of positive reference serum
- c) number of positive ELISA results at each cut-off level/number of positive faecal cultures x 100
- d) number of negative ELISA results at each cut-off level/number of negative faecal cultures x 100
- e) number of positive ELISA results which were faecal culture-positive/number of all ELISA-positives x 100
Specificity of the dot ELISA in group 1 was 17.3% at a cut-off OD of < 0.1, increasing to 96.2% at a cut-off OD of 0.4, as seen in Table I. The corresponding negative predictive value was 100%, as every animal in group 1 had tested negative by faecal culture.

The diagnostic sensitivity in group 3 was 75% at OD < 0.1, decreasing to 50% at the next cut-off value (OD = 0.1) (positive predictive value: 5.1%) on the basis of only four cattle which were + and ++ shedders (Table I). Specificity of the dot ELISA with absorbed serum was 0% at OD < 0.1, 9.8% at OD 0.1, 19.5% at OD 0.2, and 36.6% at OD 0.3.

Results with non-absorbed sera are presented in the lower half of Table I. Sensitivity for group 2 was 97.5% at OD < 0.1, 92.5% at OD 0.1, 87.5% at OD 0.2 and 85% at OD 0.3, whereas specificity for group 1 did not reach 100% until the cut-off OD value reached > 0.9, which was three cut-off values greater than the value giving 100% specificity when absorbed sera were used. In general, test specificity was higher for all groups when M. phlei-absorbed sera were used than with non-absorbed sera (Table I). However, test sensitivity was less affected by the absorption procedure.

The mean dot ELISA values were significantly higher in ++++ shedders than in +, ++ and +++ shedders. Dot ELISA values correlated with faecal culture results for ++++ shedders only, and dot ELISA sensitivity using both absorbed and non-absorbed sera was calculated for these animals alone. Sensitivity using absorbed sera in group 2 was higher among ++++ shedders (85.7% at OD 0.1) than among all shedders combined (82.5% at OD 0.1).

**DISCUSSION**

The diagnostic potential of a dot ELISA using sera absorbed with M. phlei rather than non-absorbed serum was investigated in herds with known infection status determined by faecal culture. The authors confirmed the results of Milner and colleagues (8, 9, 10), Yokomizo and colleagues (14, 15) and Lopez and colleagues (7), that the dot ELISA was as sensitive when using absorbed sera as with non-absorbed sera, while the number of false-positive reactions was significantly decreased by using absorbed sera, as shown by the gains in specificity at lower cut-off values (Table I). Serological diagnosis of paratuberculosis in cattle by visual determination was reported recently (13). This work showed that the dot ELISA gave positive results for sera from 86 of 101 (85.1%) clinically-suspect cattle testing positive by faecal culture and negative results for sera from all 64 uninfected cattle tested. The dot ELISA was also evaluated through a comparative test with faecal culture results from 262 animals belonging to three herds known to be infected with M. paratuberculosis: the false-positive rate for dot ELISA was 5 of 236 (2.1%) faecal culture-negative animals and the sensitivity was 18 of 26 (69.2%) faecal culture-positive individuals.

In the present study, paratuberculosis was diagnosed by visual determination in 29 of 44 (65.9%) cattle testing positive by faecal culture. The visual test gave negative results in 85 of 93 (91.4%) cattle testing negative by faecal culture when using non-absorbed sera. The results are compatible with those in the above study, taking into account the differences between the cattle populations tested and the fact that tests were conducted in a different laboratory. However, when absorbed sera were used in
this study, the sensitivity of visual determination decreased to 15 of 44 (34.1%) and the
specificity increased to 91 of 93 (97.8%). With regard to bacterial shedding of pathogen
tested by faecal culture, 22 of 29 (75.9%) animals testing positive by dot ELISA using
non-absorbed sera were shedding > 1,500 colonies/g of faeces at the time of serological
testing together with 11 of 15 (73.3%) of those testing positive when using absorbed
sera; thus approximately 75% of cattle testing positive by dot ELISA are heavy
shedders (> 1,500 colonies/g of faeces). The ability of the dot ELISA to diagnose
paratuberculosis infection correlated approximately with results obtained by faecal
culture in heavy shedders, but not in light shedding animals. This confirmed findings by
Milner and colleagues (11), which indicated that seroconversion occurred in infected
cattle at the onset of shedding of *M. paratuberculosis* in faeces, as detected by faecal
culture. Thus, the dot ELISA with absorbed sera could be of value if used at regular
intervals to detect heavy shedders of the organism in order that these animals may be
removed from a heavily-infected herd. A diagnostic reagent kit, "Johne’s absorbed
enzyme immunoassay", has recently become available for the detection of
paratuberculosis in cattle. This kit has a reported specificity of 99.8%, on the basis of
results obtained by testing 997 serum samples from cattle from a geographical area
known to be free of paratuberculosis. This kit has also been evaluated by an
independent laboratory in the United States of America (5), where it was recently
licensed. A test specificity of 99% was reported on the basis of testing 196 serum
samples from cattle without paratuberculosis. Results of the efforts of the authors to
develop a serological diagnostic system for Johne’s disease by using absorbed sera
support the results of the above studies.

Results obtained in the dot ELISA by both visual and objective densitometric
measurement were compatible with regard to test specificity. Visual determination was
less sensitive than objective measurement, although still acceptable compared to most
other serological tests for paratuberculosis (4).

CONCLUSIONS

Results of this study support other studies (13) in concluding that further
development of the visual dot ELISA serologic tests may be justified in an attempt to
develop a field test to assist in the diagnosis of heavy *M. paratuberculosis* shedders in
high-prevalence herds. As recently applied in African swine fever diagnosis (12),
visual dot ELISA technology would be adaptable for field testing, because the result
is determined visually and requires minimal laboratory confirmation (13).

ACKNOWLEDGEMENT

The authors wish to thank the Danish Veterinary Research Council, which partially
funded these studies with a grant in August 1990.

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Résumé : Dans cet article, les auteurs décrivent la réaction des bovins à un test immuno-enzymatique (dot enzyme-linked immunosorbent assay : dot-ELISA) utilisant des sérums absorbés avec Mycobacterium phlei. Les résultats obtenus par lecture à l’œil nu sont comparés à ceux déterminés à l’aide d’un densitomètre. Le degré d’infection des bovins a été déterminé par coproculture. L’examen a porté sur des sujets présentant différents niveaux d’exposition à l’agent pathogène et différents stades d’évolution clinique.

Les auteurs ont observé une forte augmentation de la réaction au test dot-ELISA, que ce soit avec sérums absorbés ou non absorbés, chez les malades excrétant de fortes quantités de M. paratuberculosis, par rapport à des animaux chez lesquels M. paratuberculosis était absent ou plus rare en coproculture (P < 0,05).

La paratuberculose a été diagnostiquée par lecture à l’œil nu des résultats du test dot-ELISA avec sérums non absorbés chez 29 animaux sur 44 (65,9 %) cliniquement suspects et donnant des résultats positifs en coproculture, et chez 85 sujets sur 93 (91,4 %) chez lesquels la coproculture s’est révélée négative. Avec les sérums absorbés, la sensibilité de la détermination à l’œil nu est tombée à 15 sur 44 (34,1 %), tandis que la spécificité augmentait, passant à 91 sur 93 (97,8 %). Près de 75 % des sujets, qui ont réagi positivement à l’épreuve dot-ELISA, se sont avérés être d’importants excréteurs de bactéries (> 1 500 colonies/g de fèces) lors des tests sérologiques.

Lorsqu’on compare les résultats du test dot-ELISA déterminés à l’œil nu à ceux obtenus par des données densitométriques, on aboutit à une spécificité compatible. Quant à la sensibilité du test dot-ELISA, elle est de 65,9 % pour les sérums non absorbés avec évaluation à l’œil nu et de 87,5 % par densitométrie à partir du seuil de 0,2. Pour les sérums absorbés, ces valeurs sont respectivement de 34,1 % et 82,5 %.


Resumen: Los autores describen la reacción de los bovinos a una prueba inmunoenzimática (dot ELISA) en que se usaron sueros absorbidos con Mycobacterium phlei. Los resultados obtenidos por determinación visual son comparados a los obtenidos a partir de un densímetro. El grado de infección de los bovinos se estableció mediante coprocultivo. El examen se hizo en animales que presentaban diferentes niveles de exposición a la infección y de signos clínicos.

Se observó un fuerte aumento de la reacción a la prueba dot ELISA, tanto con sueros absorbidos como no absorbidos, en los animales que excretaban gran cantidad de M. paratuberculosis, en relación con animales que
reaccionaron negativamente al coprocultivo o en quienes la excreción de M. paratuberculosis era menor (P < 0,05).

La paratuberculosis se diagnosticó a partir de la determinación visual de los resultados de la prueba dot ELISA con sueros no absorbidos en 29 animales sobre 44 (65,9%), clínicamente sospechosos y con resultados positivos en coprocultivo, y en 85 sobre 93 (91,4%) de reacción negativa al coprocultivo. Con sueros absorbidos, la sensibilidad de la determinación visual cayó a 15 animales sobre 44 (34,1%), mientras aumentaba la especificidad, pasando de 91 a 93 (97,8%). Cerca del 75% de los animales con reacción positiva a la prueba dot ELISA mostraron ser importantes excretor de bacterias cuando se llevaron a cabo las pruebas serológicas (> 1.500 colonias/g de heces).

Cuando se comparan los resultados de la prueba dot ELISA determinados visualmente con los obtenidos a partir de la medición objetiva del densímetro, se llega a una especificidad compatible. En cuanto a la sensibilidad de la prueba dot ELISA, es de 65,9% para los sueros no absorbidos con determinación visual y de 87,5% con evaluación densimétrica, a una densidad óptica de corte de 0,2; y para los sueros absorbidos, los valores respectivos son de 34,1% y 82,5%.


REFERENCES


