Use of an enzyme-linked immunosorbent assay for serodiagnosis of clinical paratuberculosis in goats. Study by Western blotting of false-positive reactions

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Summary: An enzyme-linked immunosorbent assay (ELISA) was performed for diagnosis of paratuberculosis in goats, using as antigen a protoplasmic extract (PPA-3). The test was developed on the basis of the results obtained with two serum reference pools, positive and negative respectively. To avoid day-to-day variations, dilutions of the positive serum pool were included in each plate to obtain an arbitrary system, transforming absorbance into immunoglobulin (Ig)G anti-Mycobacterium paratuberculosis units.

The ELISA was used on sera of two reference groups of animals. One group consisted of 35 goats suspected of being infected with paratuberculosis, which was confirmed by histological findings and isolation of M. paratuberculosis. The negative group consisted of 61 healthy goats from a farm free of paratuberculosis.

The test showed a sensitivity of 100% and a specificity of 91.8%. Absorption of sera with a Mycobacterium phlei suspension did not modify either the sensitivity or the specificity of the test. Sera from the negative group were analysed by Western blotting, and four of them recognised two fractions with a molecular weight of 17.3 and 28.1 kDa.

KEYWORDS: ELISA – False-positive reactions – Goats – Paratuberculosis.

INTRODUCTION

One of the most important characteristics of enzyme-linked immunosorbent assay (ELISA) methods is their ability to increase measurability of the immunological responses. They are therefore very useful methods for the diagnosis of different infections in animals, including paratuberculosis of ruminants (1, 11, 13, 16).

However, there have been few studies to evaluate the usefulness of these methods in the diagnosis of paratuberculosis in goats, in particular their usefulness at different
stages of the evolution of the disease. The stage of evolution of the disease seems to have an important effect on the sensitivity of detection methods, at least in other livestock (17).

The amplifying power of ELISA for the detection of specific antibodies sometimes leads to false-positive reactions. To avoid these false-positive reactions, some strategic methods have been developed for the diagnosis of paratuberculosis; for instance, preabsorption of test sera with a *Mycobacterium phlei* suspension (19).

The objective of the present study was the evaluation of an ELISA method used on animals in which the disease had advanced to a stage where clinical signs were manifest, as a preparatory step to other studies which will include both clinical and subclinical stages. The effect of the preabsorption technique on the results was also examined.

**MATERIAL AND METHODS**

**Sera**

Serum samples used as a control were obtained from two groups of animals. The first of these, considered positive, consisted of 35 adult goats of Murciano-Granadina and Malagueña breeds from different farms in southern Spain with a history of paratuberculosis, in which clinical signs characteristic of the disease were observed (progressive loss of weight, decrease in milk production, some diarrhoea, etc.). These animals were slaughtered and Johne's disease was diagnosed by gross and histopathological findings (3), and confirmed bacteriologically by isolation of *M. paratuberculosis* in Löwestein-Jensen medium containing mycobactin J (6).

All sera were analysed in serial twofold dilutions and their content of immunoglobulin (Ig)G antibodies assessed in an ELISA using a fixed concentration of antigen and conjugate. Four of them showed a high level of specific IgG, and their response curves were parallel to each other. The four sera were pooled and used to establish reference sero-units (12).

The group of animals considered as negative consisted of 61 healthy goats of a Canarian breed from a herd in Lanzarote (Canary Islands) with no history of paratuberculosis. Four goats were selected at random and their sera used as a negative reference pool.

**ELISA**

Both positive and negative reference pools were used to establish optimal concentration of antigen (PPA-3) (protoplasmic solution of strain 18 [Kirkergaard and Perry Laboratory]) and conjugate anti-IgG-peroxidase (9) according to results obtained by successive twofold dilutions. Finally, an antigen concentration of 50 µg/ml in carbonate/bicarbonate buffer (pH 9.6) and a dilution of 1:500 of conjugate (anti-IgG-peroxidase) in 0.1 M phosphate-buffered saline (PBS) with 5% (v/v) of inactivated newborn calf serum were employed.

Microtitre plates were incubated overnight at 4°C with 100 µl of antigen solution per well and washed four times with 0.01 M PBS solution with 0.05% of Tween. Test sera were analysed in duplicate at a single dilution 1:100 in 0.01 M PBS solution with 5% newborn calf serum. Also added to each plate was a series of six duplicate dilutions
of the reference positive serum pool (12). Test sera and pool were incubated for an hour at room temperature and washed four times with Tween-PBS buffer. Subsequently, 100 µl per well of conjugate was added and incubated and washed in the same conditions. Finally, each well received 100 µl of peroxidase substrate which consisted of 0.04% (w/v) O-phenylenediamine (OPD) and 0.012% (v/v) 33% H₂O₂ in 0.3 M citrate buffer (pH 4.7). After incubation at room temperature for 30 min, the reaction was stopped with 50 µl 3 NH₄SO₄. The optical density (OD) of each well was measured using an eight-channel photometer at a wavelength of 492 nm.

For each plate, a standard curve was prepared using the six duplicate dilutions of the positive reference pool. The OD of the highest concentration was specified as 100% bound. The OD observed on each test serum was expressed as a percentage of this value. A reference curve was constructed, plotting the percentage bound in each reference sample against the logarithm of its antibody content with the 100% bound sample being defined as containing 100 antibody units.

ELISA

The absorbed ELISA was developed following the steps described above but using sera preabsorbed with a *M. phlei* suspension as test samples. The method described by Yokomizo and colleagues (19) was followed.

Polyacrylamide gel electrophoresis and Western blotting

Proteins of *M. paratuberculosis* were separated by SDS polyacrylamide gel electrophoresis using a discontinuous system (7). Samples were run under reducing conditions on running gels of 10% concentration of acrylamide. Molecular weight standards were included in each gel.

Transfer of proteins from polyacrylamide gels to nitrocellulose paper was performed using a semi-dry system for 15 min. To determine which protein bands were recognised by antibodies from negative sera, the remaining binding sites on the nitrocellulose were blocked with 3% non-fat milk solution in PBS containing 0.1% Tween. Serum samples were diluted 1:10 in PBS containing 0.2% of bovine serum albumin (BSA), added to the nitrocellulose sheet and incubated at room temperature for 1 h on a rocking platform. Sheets were washed three times with PBS-Tween and incubated with conjugate at 1:50 dilution in PBS-BSA for 1 h. The sheets were washed again with the same buffer, and the reaction was developed using a solution of 4-chloro-1-naphthol in citrate buffer containing H₂O₂. The reaction was stopped by pouring tap water on the sheets.

Statistical methods

The discrimination value between positive and negative ELISA responses was derived from the mean plus two standard deviations of 61 samples taken from the negative group. The T test was used to study significant differences between absorbed and non-absorbed sera.

RESULTS

ELISA

After choosing the optimal concentration of antigen and dilution of conjugate, using fixed conditions and reference pool sera, each test serum was investigated for the number of IgG anti-*M. paratuberculosis* units following the method described above.
The results obtained in positive and negative groups are shown in Figures 1 and 2, where the cut-off point is represented as a horizontal line (16 sero-units). As can be seen, all sera belonging to the positive group had a level of specific IgG anti-\textit{M. paratuberculosis} units higher than the cut-off point (sensitivity 100%), but five sera from the negative group also showed a number of specific sero-units higher than 16, giving the test a specificity of 91.8%.

\textbf{Fig. 1}

\textbf{Enzyme-linked immunosorbent assay for the presence of IgG anti-\textit{Mycobacterium paratuberculosis} units in sera from goats (positive group)}

\textbf{Fig. 2}

\textbf{Enzyme-linked immunosorbent assay for the presence of IgG anti-\textit{Mycobacterium paratuberculosis} units in sera from goats (negative group)}
Absorbed ELISA

The results obtained when samples were absorbed with a *M. phlei* suspension prior to testing are shown in Figures 3 and 4 (positive and negative groups respectively). In this case, all the sera of the positive group still showed a positive ELISA (sensitivity 100%), but only four of the five sera of the negative group which showed positive results in the non-absorbed ELISA were still positive (specificity 93.4%).

**Fig. 3**
Absorbed enzyme-linked immunosorbent assay for the presence of IgG anti-*Mycobacterium paratuberculosis* units in sera from goats (positive group)

**Fig. 4**
Absorbed enzyme-linked immunosorbent assay for the presence of IgG anti-*Mycobacterium paratuberculosis* units in sera from goats (negative group)
The statistical comparison (T test) of means of results obtained with and without absorption treatment suggests that there is no significant difference between the two methods.

Western blotting

The sera of animals from the negative group were analysed using Western blotting. The only sera which developed positive reactions were the four sera of the group which showed positive absorbed ELISA results.

These four sera had antibodies which reacted with a fraction of antigen with a molecular weight of 17.3 kDa. Two sera also reacted with a 28.1 kDa band.

DISCUSSION

ELISA

The ELISA methods used in diagnosis of paratuberculosis in livestock are characterised in general by their high sensitivity and specificity (5, 11). However, these properties can be affected by different circumstances. One of the most important factors seems to be the stage of infection: this can diminish the sensitivity of the test for cows with paratuberculosis from 100% to 54.5% (17).

The same factors probably occur in caprine paratuberculosis, in such a way that the immunological methods might have variable sensitivity throughout the infection. In fact, the authors have been able to observe this phenomenon, at least in animals vaccinated with a live strain of *M. paratuberculosis*. Thus, the lymphoproliferative responses were positive far earlier than the serological responses (unpublished findings).

Should these variations in sensitivity be confirmed (depending on the level of infection or the period of time following inception), it might be necessary to use methods which study humoral responses at the same time as cellular responses, or even methods which establish the presence of micro-organisms in the faeces of infected animals (4), especially if individual diagnosis is attempted of animals from infected herds, in which a large range of levels of infection and consequently different types of immune responses might be found.

Similarly, the specificity of the test can be influenced by cross-reactions to different micro-organisms (10). This involves micro-organisms capable of stimulating immunological responses similar to those produced by the causal agent of this infection. Most notable are the species found in the genera *Mycobacterium*, especially those included in the *M. avium* complex. In fact, the antigen used in this study was obtained from a strain which is at last considered to be within the *M. avium* complex. However, the analysis of genoma of both micro-organisms reveals a high similarity, demonstrating the large number of common antigenic determinants (2). A revision of the classification of this complex has been suggested. The large differences in results reported (especially in cattle) could also be due to the use of different test methods (8, 11, 19).

With goats, previous studies showed a high sensitivity; for instance, Molina (13) found that sensitivity reached 90%, a little lower than the level observed in the present study. These differences can be explained by the procedures followed to establish the negative and positive control groups. In the 1991 study, some animals infected with *M. tuberculosis* (micro-organisms responsible for cross-reactions) were included in the
negative group, thus the cut-off point based on the mean plus two standard deviations was probably higher than the sensitivity obtained in the present study, where all the members of the negative group were apparently healthy. If some of the animals belonging to the positive group did not develop a high level of specific antibody, this would lower ELISA reactivity. In the same way, the specificity could also be affected.

The substrate used in each case could also affect the final results. In the present study, an OPD solution was used. This substrate is characterised in general by high activity, although it is also regarded as inconvenient because of its instability and its mutagenic properties.

In general, it can be concluded that the ELISA methods show a higher sensitivity and specificity in goats infected with *M. paratuberculosis*, especially when the animals are clinically affected, and more so if this is compared with other serological methods, such as those based on immunoprecipitation (15). However, some infections can develop false-positive reactions, and further study is necessary to attempt to find ways to eliminate these.

**Absorbed ELISA**

False-positive reactions seem to be the main problem in the use of ELISA methods in diagnosis of paratuberculosis. To avoid this kind of reaction, Yokomizo and colleagues (19) suggested the preabsorption of test sera with a *M. phlei* suspension. This method was used on goats by Milner and colleagues (12) and good results were obtained with respect to the specificity, which reached 100%. However, in the present study, this treatment eliminated only one of the five false-positive results obtained by non-absorbed ELISA, and statistical comparison suggested that there were no significant differences between the results for absorbed and non-absorbed sera. These results coincide with those obtained by Molina and colleagues (14) when the effect of preabsorption of sera was studied. It seems that some apparently healthy animals can develop serological responses which recognise antigenic fractions of *M. paratuberculosis* and these responses are not removed by preabsorption. However, it would be necessary to compare other methodologies of preabsorption on goat sera before definite conclusions can be reached.

**Western blotting**

The presence of false-positive reactions in ELISA methods of diagnosis of paratuberculosis has favoured the use of Western blotting, especially with cattle. Some authors have observed that sera of healthy animals react with different molecular weight proteins of *M. paratuberculosis*. For example, Milner and colleagues (11) observed that these proteins had a weight of approximately 65 kDa. Xintay and Yoghou (18), when using sera of animals artificially inoculated with different micro-organisms, observed that these reactions can be established with a high range of fractions with molecular weights of 24.6, 25.9, 40.7, 60.3 and 92.5 kDa. At the same time, some of these reactions were not prevented by preabsorption treatment. Taking these results into consideration, sera of apparently healthy goats might produce false-positive reactions with some proteins of *M. paratuberculosis*.

The results obtained by Western blotting in the present study are in accordance with those obtained with absorbed ELISA, since only the four sera with cross-reacting antibodies showed any activity. This activity was represented by specific reactions with polypeptides of 17.3 and 28.1 kDa.

Preabsorption did not modify these results, which is in accordance with results obtained by Molina (13) using a similar procedure.
CONCLUSION

In conclusion, the specificity of ELISA methods could be affected by the presence in test sera of antibodies which recognise antigenic fractions of *M. paratuberculosis* with molecular weights of 17.3 and 28.1 kDa, and these cross-reactions are not removed by preabsorption with a *M. phlei* suspension. However, it is necessary to develop comparative studies between different preabsorption methodologies, which include the elimination of both polypeptides.

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Résumé: Une épreuve enzyme-linked immunosorbent assay (ELISA) utilisant pour antigène un extrait de protoplasme (PPA-3) a été appliquée au diagnostic de la paratuberculose chez les caprins. Le test a été réalisé à partir des résultats obtenus avec deux mélanges de sérums de référence, l’un positif et l’autre négatif. Pour éviter les variations au jour le jour, des dilutions du sérum positif ont été déposées sur chaque plaque afin d’obtenir un système arbitraire, transformant la densité optique en unités de l’immunoglobuline (Ig)G anti-Mycobacterium paratuberculosis.

La technique ELISA a été appliquée aux sérums de deux groupes d’animaux de référence. L’un de ces groupes comprenait 35 chèvres suspects, chez lesquelles la paratuberculose a été ensuite confirmée par examen histologique et isolement de *M. paratuberculosis*. Le groupe négatif était constitué de 61 chèvres saines provenant d’un élevage exempt de cette maladie.

Le test a présenté une sensibilité de 100 % et une spécificité de 91,8 %. L’absorption des sérums avec une suspension de *M. phlei* n’a modifié ni la sensibilité ni la spécificité du test. Les sérums du groupe négatif ont été analysés selon la technique du Western blot ; quatre d’entre eux ont reconnu deux fractions d’un poids moléculaire de 17,3 et 28,1 kDa.


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Resumen: Se ha utilizado un test ELISA para el diagnóstico de la paratuberculosis en cabras, usando como antígeno un extracto protoplasmico (PPA-3). El test se desarrolló en función de los resultados obtenidos sobre dos «pools» séricos de referencia, positivo y negativo respectivamente. A fin de
eliminar posibles variaciones de un día a otro, se incluyeron en cada placa distintas diluciones del pool de referencia positivo, obteniendo con ello un sistema arbitrario mediante el que los valores de absorbancia fueron transformados en unidades IgG anti-Mycobacterium paratuberculosis.

El test se empleó sobre el suero de dos grupos de animales de referencia. Uno de ellos estuvo formado por 35 cabras sospechosas de padecer paratuberculosis, infección que fue confirmada por los hallazgos histopatológicos y el aislamiento de M. paratuberculosis. El grupo negativo estuvo formado por 61 cabras sanas procedentes de una granja libre de paratuberculosis.

El test mostró una sensibilidad del 100%, y una especificidad del 91,8%. La absorción de los sueros con una suspensión de M. phlei no modificó ambos parámetros. Finalmente, los sueros del grupo negativo fueron analizados por Western-blotting, y cuatro de ellos reconocieron dos fracciones proteicas con un peso molecular de 17,3 y 28,1 kDa.


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REFERENCES


