Advances in the diagnosis of some parasitic diseases by monoclonal antibody-based enzyme-linked immunosorbent assays

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Summary: Advances in diagnostic assays for parasitic diseases include the use of monoclonal antibodies (MAbs) in antigen capture and competitive inhibition enzyme-linked immunosorbent assays (C-ELISA). Antigen capture ELISAs for Anaplasma marginale and Cryptosporidium parvum provide direct detection of these parasites during clinical disease, and the C-ELISA format has been adapted for detection of anti-Babesia equi, anti-A. marginale and anti-bluetongue virus antibodies. False-positive results may occur when antigen preparations in other ELISA formats are contaminated with Escherichia coli, erythrocyte or cell-culture antigens. The C-ELISA format overcomes problems of antigen purity, since the specificity of the C-ELISA depends solely on the MAB used. For this reason, the C-ELISA format is highly suited for use with recombinant antigens. Also, the use of recombinant protein in diagnostic assays precludes the need to infect animals for antigen production when the antigen cannot be produced in cell culture.

KEYWORDS: Anaplasma marginale - Antigen capture ELISA - Babesia equi - Bluetongue virus - Competitive inhibition ELISA - Cryptosporidium parvum - Recombinant proteins.

INTRODUCTION

New diagnostic assays are developed to improve current tests or to provide an assay when one is not available. When a new diagnostic assay is developed as a replacement for a current diagnostic test, methods must be found to provide accurate comparative test performance (sensitivity and specificity) data. A common method of evaluation is to simply compare the results of the new assay with those of the test currently in use. This comparison results in quantification of the agreement (concordance) between the two assays but does not address the question of differences in sensitivity or specificity. Sensitivity is defined by the following formula:

\[
\text{Sensitivity} = \frac{\text{test positives} \times 100}{\text{test positives} + \text{false negatives}}
\]
and specificity is defined as follows:

\[
\text{specificity} = \frac{\text{test negatives} \times 100}{\text{test negatives} + \text{false positives}}
\]

It is clear from these formulas that sensitivity measures false-negative results and specificity quantitates false-positive results (23).

Much of the difficulty in obtaining accurate test performance data resides in defining a “gold standard” for disease in the above formulas. One such accepted “gold standard” is the transmission of disease from an animal which is test positive to an animal which is test negative. For most experimental designs, transmission data cannot be obtained for every animal tested. Depending on the reasons for developing a new diagnostic assay, concordance data may be a sufficient first step in test evaluation. For example, if a new diagnostic assay is designed solely to provide a more rapid test, concordance data may be sufficient for validation. If a test with improved performance is required, then the best “gold standard” for comparison must be identified and utilized for validation of a new assay. Clearly, when a diagnostic assay is the first test reported for a particular disease, a “gold standard” must be identified which can provide the criteria for test validation. In this context, some recently-developed monoclonal antibody (MAb)-based enzyme-linked immunoabsorbent assays (ELISAs) are reviewed.

**ANTIGEN CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY**

The primary advantage of the antigen capture ELISA is the ability of this test to detect parasite antigen directly from an animal prior to or during clinical disease. The main component of the antigen capture ELISA is a MAb. The desired characteristics of this MAb are:

- strong binding to the parasite
- the ability to attach to an ELISA plate without loss of reactivity
- in addition, a second MAb recognizing an epitope other than the MAb which is bound to the ELISA plate is often used as part of the indicator system.

Anaplasmosis, a vector-borne haemoparasitic rickettsial disease, is caused by *Anaplasma marginale* (20) and *A. centrale* (21). Clinical disease is characterized by anaemia, weight loss, abortion and death (1), and survivors are lifelong carriers of the rickettsia (19). Recently, an antigen capture ELISA has been developed for the detection of *A. marginale* rickettsaemia prior to the onset of clinical disease (22). The assay sensitivity is 1.1(±0.5)% parasitized erythrocytes. This ELISA uses two MAbs which recognize different epitopes on a major surface protein of *A. marginale* (22). These epitopes had previously been shown to be conserved among different *A. marginale* isolates (17). The ELISA was validated by testing blood from twenty-four calves before and after experimental infection with *A. marginale* (22). This assay was shown to be specific for *A. marginale*, in that blood samples containing *Babesia bovis*, *B. bigemina*, *A. ovis* and six antigenic variants of *Trypanosoma brucei* tested negative (22).

Since this assay discriminates between anaplasmosis and other clinically similar haemoparasitic diseases, it permits diagnosis and treatment of cattle in a critical stage of infection (22). A primary advantage of this assay is the detection of infected cattle early
in the course of disease, when tetracycline therapy is most successful in preventing death (16).

Cryptosporidiosis (caused by the sporozoan Cryptosporidium parvum) is a common infection of young calves (4). C. parvum is one of the major causes of neonatal diarrhoea in calves and contributes to significant economic losses in the beef and dairy cattle industries (9). An antigen capture ELISA which detects Cryptosporidium sp. oocysts in bovine faeces was recently described (3). For capture, the assay uses an immunoglobulin (Ig)M MAb which binds to a 40 kDa oocyst antigen, and uses polyclonal goat anti-oocyst serum in the indicator system (3). The format of this test is illustrated in Figure 1.

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**HRPO**

**Rabbit anti-goat Ig, HRPO conjugate**

**Goat anti-oocyst**

**C. parvum oocysts**

**MAb anti-oocyst**

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**HRPO**: horseradish peroxidase  
**Ig**: immunoglobulin

**FIG. 1**

**Schematic representation of a monoclonal antibody (MAb) capture enzyme-linked immunosorbent assay used for Cryptosporidium parvum oocysts**

Courtesy of Dr L.E. Perryman, Washington State University, Pullman, Washington, United States of America

(3)

The assay detects as few as $3 \times 10^5$ C. parvum oocysts per ml of faeces and does not detect oocysts of *Eimeria auburnensis*, *E. bovis*, *E. ellipsoidalis* or *E. zuernii* (3). The assay was validated by:

1. testing faeces from naturally or experimentally-infected calves from three locations
2. comparing the ELISA to indirect immunofluorescence and light microscopic analysis
3. following the kinetics of faecal shedding of C. parvum oocysts by experimentally-infected calves (3).
The utility of this assay is that it is sufficiently sensitive to detect oocysts in animals with clinical signs of disease, as well as in calves which have recovered from diarrhoea but which continue to shed oocysts (3). Furthermore, this ELISA has been used to detect *C. parvum* oocysts in diarrhoeic faeces from llamas and young horses with severe combined immunodeficiency (3).

**COMPETITIVE INHIBITION ELISA**

In addition to the detection of parasites during clinical stages of infection, it is also necessary to identify animals which have survived clinical disease and which are persistent carriers. Parasite levels in carrier animals are often too low to be detected by antigen capture ELISA; however, specific antibody is often maintained at detectable levels. First described for use in detecting anti-bluetongue virus antibody (2), the MAb-based competitive inhibition ELISA (C-ELISA) format has been used to detect antibodies to *Cowdria ruminantium*, *A. marginale* and *Babesia equi* (10, 11, 24). A C-ELISA for detection of bovine anti-bluetongue virus antibody is commercially available (18).

Components of the C-ELISA include a MAb and the corresponding epitope. The MAb must possess an appropriate binding affinity such that polyclonal antibody can replace it in the competitive reaction. Desirable epitope characteristics include a linear peptide composition, immunodominance, and conservation among isolates. The latter is an important characteristic in cases where production of the epitope by molecular cloning and expression in bacteria is necessary. Since the specificity of the C-ELISA depends entirely on the MAb used, the test is well suited for use with recombinant antigens. If the recombinant antigen is expressed in sufficient quantity to provide an appropriate signal:noise ratio after application to the ELISA plate as a bacterial lysate, additional purification of the recombinant antigen is not necessary.

The complement fixation (CF) test is currently the official test for anti-*B. equi* antibodies in many countries, including the United States of America (5, 6, 8). However, the test still has a number of limitations, as follows:

a) CF cannot be used to test sera with anti-complement activity or sera which react with the control erythrocyte antigen used in the test

b) sera containing specific IgG(T) antibody may yield false-negative results because IgG(T) does not fix complement by the classical pathway (14)

c) the CF test has been shown to yield variable results in persistently-infected horses (7).

A MAb-based C-ELISA for detection of equine anti-*B. equi* antibodies has been described recently (11). The format of this ELISA is illustrated in Figure 2. This C-ELISA uses an IgGl MAb which binds to a peptide epitope on the surface of *B. equi* merozoites (12). A concordance of 94% was found when 154 sera from 19 countries were tested by the C-ELISA and CF (11). In this study, sera yielding discrepant results between the CF test and C-ELISA were retested by immunoprecipitation of 35S-methionine-labelled *in vitro* translation products of messenger ribonucleic acid from *B. equi* (11, 12). Immunoprecipitation results indicated that sera which tested negative by CF and positive by C-ELISA were positive for *B. equi* (11). The C-ELISA was shown to be specific for anti-*B. equi* antibody, as horses infected with *B. caballi* tested...
Illustration of monoclonal antibody (MAb)-based competitive inhibition enzyme-linked immunosorbent assay for *Babesia equi*
(12)

Courtesy of Dr Elizabeth S. Visser, Onderstepoort Veterinary Institute, Onderstepoort, South Africa

When equine test serum contains specific antibody, the specific equine antibody competes with the mouse MAb, thus decreasing the optical density (OD) to significantly below the level given by the negative reference serum, and the equine serum is considered positive. When the equine test serum does not contain specific antibody, the mouse monoclonal antibody binds to the epitope, thus yielding an increased optical density equivalent to negative reference serum, and the equine serum is considered negative.
negative (11). The advantage of the C-ELISA is that the format overcomes limitations a) and b) listed above. Furthermore, the *B. equi* protein bearing the epitope used in the C-ELISA has been molecularly cloned and expressed in *Escherichia coli*, and the C-ELISA has been reformatted with this antigen (13).

Recently, a highly-conserved *A. marginale* major surface protein (MSP-5) was described (24). In preliminary testing, MSP-5 and a corresponding MAb successfully detected anti-*A. marginale* antibody in immune bovine sera (24). In addition, another *A. marginale* major surface protein (MSP-3) has demonstrated potential as a diagnostic antigen in the detection of anti-*A. marginale* antibody in the sera of long-term carrier cattle (15).

**CONCLUSIONS**

MAb-based ELISAs have tremendous potential for use in the diagnosis of parasitic diseases. The antigen capture ELISA can directly detect the presence of a parasite during the clinical stages of disease when treatment is most efficacious, and the C-ELISA format can be used to detect carriers of persistent parasitic infections. A final and important attribute of the C-ELISA is the ease with which it can be used with recombinant antigens. This is especially important in those parasitic diseases for which diagnostic antigen cannot yet be produced in cell culture. Furthermore, the production of recombinant antigen in *E. coli* precludes the necessity of infecting animals for antigen production.

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**PROGRÈS ACCOMPLIS DANS LE DIAGNOSTIC DE CERTAINES PARASITOSES GRÂCE AUX TECHNIQUES IMMUNO-ENZYMATIQUES BASÉES SUR LES ANTICORPS MONOCLONAUX. – D.P. Knowles Jr et J.R. Gorham.**

Résumé : L'utilisation des anticorps monoclonaux dans les techniques d'immunocapture enzymatique (enzyme-linked immunosorbent assay : ELISA) et d'ELISA «compétition/inhibition» (C-ELISA) a permis de progresser dans la mise au point de tests de diagnostic pour les maladies parasitaires. Les tests d'immunocapture ELISA pour *Anaplasma marginale* et *Cryptosporidium parvum* permettent de reconnaître directement ces parasites pendant la phase clinique de la maladie, tandis que le test de C-ELISA a été adapté à la détection d'antigènes *anti-Babesia equi, anti-A. marginale et anti-virus de la fièvre catarrhale du mouton*. Avec d'autres tests ELISA, on aboutit parfois à des erreurs par excès, lorsque les préparations d'antigènes sont contaminées par *Escherichia coli*, des érythrocytes ou des antigènes de culture cellulaire. Le test C-ELISA résout le problème de la pureté de l'antigène, puisque la spécificité du test ne dépend plus que de l'anticorps monoclonal utilisé. Aussi, est-il très recommandé d'utiliser ce test C-ELISA avec des antigènes recombinants. De plus, grâce aux protéines recombinantes employées dans les tests de diagnostic, il n'est plus nécessaire d'infecter des animaux pour obtenir des antigènes lorsque ceux-ci ne peuvent être produits par culture cellulaire.
Resumen: El uso de anticuerpos monoclonales en las técnicas de inmunocaptura enzimática (ELISA) o de «competición/inhibición» ELISA (C-ELISA) permitió importantes avances en el establecimiento de pruebas de diagnóstico para las parasitosis. Las pruebas de inmunocaptura ELISA para Anaplasma marginale y Cryptosporidium parvum permiten el reconocimiento inmediato de estos parásitos durante la fase clínica de la enfermedad; la prueba C-ELISA se ha adaptado para la detección de anticuerpos anti-Babesia equi, anti-A. marginale y anti-virus de la fiebre catarral ovina. Con otras pruebas ELISA pueden darse resultados falsamente positivos, cuando las preparaciones con antígenos están contaminadas por Escherichia coli, eritrocitos o antígenos de cultivo celular. La prueba C-ELISA resuelve el problema de la pureza del antígeno, ya que su especificidad sólo depende del anticuerpo monoclonal utilizado; es por lo tanto muy recomendable usar esta prueba con antígenos recombinantes. Por otra parte, gracias a las proteínas recombinantes que se usan en las pruebas diagnósticas, ya no es necesario infectar animales para obtener antígenos cuando éstos no se pueden producir por cultivo celular.

PALABRAS CLAVE: Anaplasma marginale - Babesia equi - Cryptosporidium parvum - Proteínas recombinantes - Pruebas de competición/inhibición ELISA - Pruebas de inmunocaptura ELISA - Virus de la fiebre catarral ovina.

REFERENCES


