*Full Length Research Paper*

# Comparison of cELISA and IFA tests in the serodiagnosis of anaplasmosis in cattle

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This study was performed to compare indirect fluorescence antibody (IFA) test with competitive ELISA (cELISA) for detection of antibodies to *Anaplasma marginale* in cattle. In this study, a total of 484 cattle were examined on farms that had a positive history of anaplasmosis. Thin blood smears were prepared from each examined cattle and the cELISA and IFA tests were performed. Samples in which the results of microscopic examination and cELISA were compatible were used as a "gold standard" to define sensitivity and specificity of IFA test and these were used to give "real diagnosis". According to the test result; 62 of 69 samples found positive in "real diagnosis" were positive in IFA test, and all of the 187 samples that were negative in the real diagnosis were defined as negative in IFA test. When compared to gold standard the sensitivity and specificity of the IFA test were 88.95 and 100%, respectively. In conclusion, both IFA and cELISA tests can be used in the diagnosis of *A. marginale*.

**Key words:** Anaplasma marginale, IFA test, cELISA.

## INTRODUCTION

Bovine anaplasmosis occurs in tropical and subtropical areas throughout the world and the disease is a major constraint to cattle production in many countries (Kocan et al., 2010). The existence of acute and sub-clinical infections in cattle caused by anaplasmosis has been reported in Turkey, which is located in subtropical climate zone (Birdane et al., 2006). The species that cause anaplasmosis in cattle are *Anaplasma marginale* and *A. centrale.* While *A. marginale* is a pathogen, and causes infections, *A. centrale* is less pathogenic and rarely causes clinical infection (McElwain, 2000; Parola and Raoult, 2001). *A. marginale* can be transmitted both mechanically by biting flies or blood-contaminated fomites, and biologically by ticks (Kocan et al., 2010).

Anaplasmosis can be seen at any age group in cattle. However, the severity and death rate increases with the advance in age. In terms of signalment, clinical anaplasmosis is more commonly encountered in cattle older than 1 year of age (Jones et al., 1968). Over 2 years old, clinical symptoms may include abortion, icterus, fever and often death in animals (Kocan et al., 2004). The animals that recovered clinically are the life long carriers of the agent and they are porter of the disease (Kocan et al., 2000; Stokka et al., 2000; Tassi et al., 2002).

Diagnosis of bovine anaplasmosis is usually based on signalment and presenting clinical signs, necropsy findings observed in infected animals, and the season (Jones and Brock, 1966). In order to confirm the diagnosis, laboratory tests such as light microscopic evaluation of Giemsa-stained blood smears or serological/molecular diagnostic procedures are required. In carrier animals, microscopic diagnosis can be difficult, owing to variable parasitemia, and thus, a variety of serologic tests or genetic material of the agent are used to detect the specific antibodies. Various serological and molecular techniques have been developed for this purpose. For the detection of antibodies complement fixation (CF), Card agglutination (CA) and ELISA tests are routinely performed in various laboratories (Amerault and Roby, 1968; Amerault et al., 1972; Goff et al., 1990).

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A competitive ELISA (cELISA) has been used for diagnosis of *A. marginale* infection in various ruminants including cattle, sheep and deer (Figueroa et al., 1993; Gale et al., 1996; Ge et al., 1997; Torini de Echaide et al., 1998; McElwain, 2004; de la Fuente et al., 2004). The cELISA currently used for diagnosis of bovine anaplasmosis is based on use of a monoclonal antibody (Mab) ANAF16C1 that recognizes MSP5 in *A. marginale*, *A. centrale* and *A. ovis*. The MSP-5 antigen is conserved among all known species of *Anaplasma* (Visser et al., 1992). In a study (Herrero et al., 1998) where PCR and cELISA were compared for the diagnosis of antibodies specific to *Anaplasma* in cattle sera, it has been stated that these two tests are in accordant with each other.

The indirect fluoresan antibody (IFA) test is one of the serological tests that have been widely used throughout history, for the diagnosis of many blood protozoon and rickettsia infections. The IFA test is a commonly used test in seroepidemiological researches and the cost of this test is lower than that of other serologic tests (de Kroon et al., 1990; Hungerford and Smith, 1997).

Although, many different methods have been reported for the diagnosis of *A. marginale* in cattle, to the best of our knowledge, no comparative study that evaluates the use of cELISA and IFA tests in the diagnosis of *A. marginale* has been published. The main objective of this study was to compare cELISA and IFA tests in the serodiagnosis of *A. marginale* in cattle, and to determine their concordance with microscopic examination.

## MATERIALS AND METHODS

### Animals used in the study

In this study, a total of 484 cattle from different age groups (0 to 36 months) were examined on farms that had a positive history of anaplasmosis.

### Blood smear examination

Thin blood smears were prepared from ears of each examined animal. The smears were fixed with methyl alcohol and stained with 10% Giemsa, and then washed under regular tap water and dried at room temperature. Giemsa stained thin blood smears were examined under oil immersion objective of microscope. A total of 20 to 25 fields were examined randomly from each slide for the presence of parasites and the percentage of infected erythrocytes was counted.

### Competitive-ELISA test

For use in serological tests, 10 ml blood was taken from vena jugularis of each animal and the sera were subjected to *cELISA* to determine the presence of specific antibodies *to A. marginale*. The cELISA test was performed according to the test procedure of the manufacturer (Anaplasma antibody test kit, cELISA, VMRD, Inc., USA).

### Preparation of antigen slides and test procedures for IFA test

The sera that were defined as positive and negative by microscopic examination and cELISA test were also examined with IFA test. The IFA test antigens were prepared from the blood of cattle with acute anaplasmosis that have high level of parasitemia. For this purpose, the blood was washed with diluted PBS solution after being centrifuged 3 times at 2100 rpm. A 5 µl drop of blood was added to each well of the multi-test slides. After drying at room temperature, these slides were dried with a paper towel and then packed with aluminum foil and were kept at -80°C until use. For the IFA test, 1/80 serial dilutions in PBS of cattle sera were produced and test was performed as described previously (McElwain, 2000). Anti-bovine conjugate (Sigma F7887, Anti-Bovine IgG (whole molecule) - FITC antibody produced in rabbit) was diluted 1:120 with PBS that contained 0.2% Evans blue. The existence of antigen-antibody reaction was evaluated using a fluorescence microscope (Olympus BX50, Japan). The data obtained were compared with the results of the cELISA and microscopic examination.

The sera that were used as a positive control in the IFA test were positive on the cELISA and the agent had been determined in blood smears on microscopic examination. Sera determined as negative in both examinations, were used as negative controls for the IFA test. The acuity (sharpness) of the fluorescence reaction was evaluated as a semi-quantitative interval between +1 and +5.

### Statistical analysis

To define sensitivity and specificity of IFA test, samples in which the results of microscopic examination and cELISA were in agreement were used as the "gold standard".

## RESULTS

### Determination of cut off value for IFA test

In order to define positive cut off for the IFA test, 20 negative control sera were diluted twice with PBS, starting from 1/40. When each of these samples was subjected to IFA test and antigen reaction was analyzed, it was seen that non-specific fluorescence signals were dense, at dilutions of 1/320 and below. Therefore, it was decided that samples at dilution steps higher than 1/320 be evaluated as positive.

### Comparison of IFA and cELISA Tests

In this study, blood samples from 484 cattle were examined. Comparison of microscopic examination results according to the cELISA is given at Table 1. "Gold standard" samples in which the results of microscopic examination and cELISA were concordant were used to define sensitivity and specificity of IFA test and these were evaluated as "real diagnoses". That means that, a total of 256 sera were subjected to IFA test. Comparison of IFA test results with the gold standard is given in Table 2. The sensitivity of cELISA test was at 87.3%, while specificity of cELISA test was at 100%. However,

**Table 1.** Compare microscopic examination results according to the cELISA.

| | | cELISA | | |
|---|---|---|---|---|
| | | + | - | Total |
| Microscopic examination | + | 69 | 10 | 79 |
| | - | 218 | 187 | 405 |
| Total | | 287 | 197 | 484 |

**Table 2.** Compare IFA test results according to the gold standard.

| | | Gold standard | | |
|---|---|---|---|---|
| | | + | - | Total |
| IFA test results | + | 62[a] | 0[b] | 62 |
| | - | 7[c] | 187[d] | 194 |
| Total | | 69 | 187 | 256 |

a; positive examples of the IFA test according to the real diagnose; b; as the examples of false positive IFA test (false positive); c; as the examples of false negative IFA test (false negative); d; negative examples of the IFA test according to the real diagnose.

sensitivity and specificity of IFA test were 90.7 and 100%, respectively.

## DISCUSSION

Bovine anaplasmosis occurs in tropical and subtropical areas throughout the world, and the disease is a major constraint to cattle production in many countries. In scanning tests that are used to determined prevalence of any disease in epidemiological studies, reliability of the test is crucial. In order to define whether the used test is a reliable sensitivity, validity rates such as specificity, inaccurate negativeness, inaccurate positiveness and accuracy are calculated and it is required that these measurements have adequate level. "Gold standard" samples in which the results of microscopic examination and cELISA (Table 1) were compatible were used to determine sensitivity and specificity of IFA (Table 2) test and these were evaluated as "real diagnose".

The sensitivity and specificity of cELISA and IFA tests were very high. For the detection of antibodies, complement fixation (CF), card agglutination (CA) and ELISA tests are routinely performed in various laboratories (Amerault and Roby, 1968; Amerault et al., 1972; Goff et al., 1990). Nevertheless, the reports on the sensitivity and specificity of these tests are not consistent with each other (Bradway et al., 2001). Although, DNA-based diagnostic methods could be used to identify the pathogen species of *Anaplasma* infections, a serologic test based on MSP5 would be more practical for the diagnosis of large number of animals (de la Fuente et al., 2005). It has been stated that cELISA test has very high sensitivity and specificity in the diagnosis of antibodies

shaped against *A. marginale* and it can diagnose these antibodies 6 years after infection (Visser et al., 1992; Knowles et al., 1996; Torini de Echaide et al., 1998). In a recent comparison between the competitive inhibition ELISA (CI-ELISA) and a nested PCR assay in an *A. marginale* endemic herd in the USA (Torini de Echaide et al., 1998), the sensitivity and specificity of the CI-ELISA were reported to be 96 and 95%, respectively. Several researchers have reported that IFA test may be used instead of PCR, CF and ELISA (Goff et al., 1990; Silva et al., 2006; Cantu et al., 2008). IFA test is a commonly used test in seroepidemiological research and the cost of this test is lower than that of the other serological tests. This is the first study that has compared the cELISA and IFA test in the diagnosis of *A. marginale*.

## CONCLUSION AND SUGGESTIONS

In order to determine the existence or absence of various diseases adequately, reliable tests are required. For a certain disease, methods appropriate to the conditions of the evaluation are preferred. Factors such as; whether the test is appropriate to the field and laboratory; whether it is expensive or cheap; whether the test results are obtained in a short time or not, can be a matter of choice on which test will be used.

IFA is an economical and easy method to perform. In this research, the acuity (sharpness) of the fluorescence reaction was evaluated as semi-quantitative between +1 and +5 interval. However, the test has some disadvantages because the results vary between individuals, being subjective, and consuming too much time. During the evaluation process of this test, the

knowledge and experience of the staff become important. The biggest advantage of the ELISA method is that, many sera samples (at least 90) can be examined on one micro plate at the same time. The duration of the test procedure is quite short (145 min). The results are read by an ELISA reader and quantitative values can be obtained very rapidly.

In this study, though no meaningful difference between cELISA and IFA tests was found, the fact that IFA test requires special laboratory conditions and fluorescence microscope may create difficulties for the usage of this test. Some advantages of cELISA, such as its ease of use, cheapness and the possibility to give quantitative results may be seen as a matter of choice in these kinds of researches.

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