TÜBİTAK

Research Article

Turk. J. Vet. Anim. Sci.
2012; 36(2): 131-136
© TÜBİTAK
doi:10.3906/vet-1012-632

A retrospective study of *Anaplasma* in Minnesota cattle

Aneela Z. DURRANI[1,*], Sagar M. GOYAL[2]

[1]Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Outfall Road, Lahore-PAKISTAN

[2]Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, Minneapolis and St. Paul, MN-USA

Received: 14.12.2010

Abstract: To determine the seroprevalence of *Anaplasma* in Minnesota cattle, the database of the Veterinary Diagnostic Laboratory (VDL), University of Minnesota was searched over a 10 year period (2001 to July 2010). A total of 438,407 bovine serum samples were tested by complement fixation test (CFT) and competitive enzyme linked immunosorbent test (cELISA). The positive rate of seroprevalence by CFT was 2.4% out of 211,484 samples, with a 0.13% anticomplementary result. The VDL at the University of Minnesota started using cELISA after 2002, and CFT application was suspended as diagnostic test for anaplasmosis after 2005 due to its complexity. With cELISA, positive seroprevalence was 6.8% out of 226,923 samples. The results suggest that the cELISA test was more rapid and less complex than the CFT.

Key words: Cattle, *Anaplasma*, CFT, cELISA test

Introduction

Anaplasmosis is a rickettsial disease affecting cattle in most tropical, subtropical, and temperate countries (1). *Anaplasma* was originally regarded as a protozoan parasite, but later research showed that it lacks the attributes to justify this description. Since 1957, *Anaplasma* has been classified in the family *Anaplasmataceae* of the order Rickettsiales. Based on a combination of 16S ribosomal RNA, groESL, and surface protein gene sequence analysis (2), *Anaplasmataceae* has been reorganized and now includes the genera *Ehrlichia*, *Anaplasma*, *Cowdria*, *Wolbachia*, and *Neorickettsia* (3). One of the many species of *Anaplasma*, *A. marginale* is a pathogen principally of cattle but is not confined to cattle (4).

Anaplasma is transmitted mechanically by lice, biting flies, and fomites and biologically by various tick species. Experimental transmission of anaplasma has been demonstrated with a number of species of *Tabanus* (horseflies) and with mosquitoes of the genus *Psorophora* (5). Transmission of *A. marginale* has been demonstrated with adult ticks *(Dermacentor occidentalis)* (6). Since adults of this tick species normally feed on both deer and cattle, it is probable that deer-to-deer, deer-to-cattle, and cattle-to-deer transmission occurs. This wildlife reservoir of infection (6) has significant implications for California, where it effectively negates the control of anaplasmosis by the conventional methods (test, segregation, and treatment) that are effective elsewhere. Of the 3 species of deer in the US, the

* E-mail: aneela_nadeem8@hotmail.com

black-tailed deer appears to be the most susceptible to *A. marginale* (7).

Clinical disease is most notable in cattle, but other ruminants including water buffalo, bison, African antelope, and mule deer can become persistently infected with *A. marginale* (8). The disease is characterized by fever, severe anemia, jaundice, brownish urine, loss of appetite, dullness or depression, rapid deterioration of physical condition, muscular tremors, constipation, yellowing of the mucous membrane, and labored breathing (9).

The bacterium *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) may cause infection in several animal species, including humans. The disease in domestic ruminants is also called tick-borne fever (TBF) and has been known for at least 200 years. In Europe clinical manifestations due to *A. phagocytophilum* have been recorded in sheep, goats, cattle, horses, dogs, cats, roe deer, reindeer, and humans. However, mammalian seropositive and PCR-positive results have been detected in several other species. Investigations indicate that the infection is prevalent in *Ixodes ricinus* areas in most countries in Europe. *A. phagocytophilum* infection may cause high fever, cytoplasmatic inclusions in phagocytes, and severe neutropenia but is seldom fatal unless complicated by other infections. Complications may include abortion and impaired spermatogenesis for several months. However, the most important aspect of the infection, in sheep, is its implication as a predisposing factor for other infections. Factors such as climate, management, other infections, and individual conditions are important for the outcome of the infection. *A. phagocytophilum* may cause persistent infection in several species. Based on the 16S rRNA gene sequences several variants exist. Different variants may exist within the same herd and even simultaneously in the same animal. Variants may behave differently and interact in the mammalian host (10)

Detection of persistently infected cattle is an important tool for controlling the movement of infected cattle to non-endemic regions. Microscopic examination of Giemsa stained blood smears, which are used to confirm acute anaplasmosis, can only detect levels of $>10^6$ infected erythrocytes per milliliter (11). Serological tests, including complement fixation and card agglutination, have been the most commonly used methods to detect *Anaplasma*-infected cattle in the field (12). In addition, the immunofluorescent-antibody test (IFA) and enzyme linked immunosorbent assay (ELISA) have been utilized for epidemiological studies (13).

A commercial competitive ELISA (VMRD, Pullman, WA, USA) based on serum antibody inhibition of MAb AnaF16C1 binding to rMSP5 has been developed. The competitive enzyme linked immunosorbent test (cELISA) has a demonstrated specificity of 100% with sera from uninfected cattle in regions where anaplasmosis is not endemic (14). Cattle sera received at the Minnesota Veterinary Diagnostic Laboratory (VDL) over the past 10 years were tested for antibodies using the cELISA and CFT.

Materials and methods

Competitive enzyme linked immunosorbent assay (cELISA): Serum samples from 226,923 cattle submitted during 2002-2010 were tested by cELISA. The test was performed as described (VMRD, Pullman, WA, USA). The sera, along with the positive and negative controls (70 µL each), were loaded on the coated adsorption plate. The plate was incubated for 30 min at 25 °C. The adsorbed serum samples (50 µL each) were transferred to the corresponding wells of the *Anaplasma* antigen coated plate. The uncovered plate was incubated for 60 min at 25 °C, and the wells were washed twice with diluted wash solution (200 µL/well, each wash). Antibody-peroxidase conjugate diluted with conjugate diluting buffer (50 µL) was added to each well. The plate was incubated for an additional 20 min at 25 °C. After washing the plate 4 times, 50 µL of substrate solution was added to each well. The plate was incubated for 20 min followed by the addition of 50 µL stop solution to each well. Well contents were mixed gently by tapping the side of the plate several times, and optical density (OD) was read at 650 nm; the reader was blanked on air and read plate(s). The mean OD of the negative control ranged from 0.40 to 2.10 while the percent inhibition of the positive control was ≥30%. The percent inhibition was calculated by the following formula:

% inhibition = 100 − [(Sample OD × 100)/(Mean Negative Control OD)].

Test sera having <30% inhibition were considered negative while those with ≥30% inhibition were recorded as positive.

Complement fixation test (CFT): Serum samples from 211,484 cattle submitted from 2001 to 2005 were tested by CFT. The test was performed as described by the National Veterinary Services Laboratory (NVSL), United States Department of Agriculture (1986). Vernol buffer diluents (VBD), pH 7.3-7.4, were prepared for use throughout the test. Antigen for *Anaplasma* was obtained from NVSL and diluted with VBD. Stock solution of hemolysin (Colorado Serum Co., Denver, CO, USA) was prepared by adding 0.5 mL of 1:5000 hemolysin to 49.5 mL of VBD. Working dilution of hemolysin was made by adding 0.1 mL of stock solution to 4.9 mL of VBD. Working dilutions of hemolysin and sheep RBCs were mixed in equal parts and placed in a 37 °C water bath for 10-15 min before use. Commercially available guinea pig complement (Colorado Serum Co., Denver, CO, USA) was used. Titration for hemolysin (1:100 to 1:320,000 dilutions) and complement (1:250 dilution) was done before the test. Sheep RBCs in Alsever's solution were obtained from Wilfer Laboratories, Stillwater, MN, USA. After standardizing the 2% suspension of sheep RBCs at 540 um, an OD of 0.600 $\pm$ 0.500 was considered acceptable. A total of 10 mL of sensitized RBCs with hemolysin were used for each 192 sample batch; 2 positive (read +4 at 1:5 dilutions) and 3 negative control sera (NVSL, United States Department of Agriculture) were used with each plate. After dilution with VBD the test sera were deactivated at 58 °C for 35 min. Deactivated serum (0.025 mL), along with 0.025 mL of antigen and 0.025 mL of diluted complement, was added to each plate well. After incubation of plate at 37 °C for 1 h, 0.05 mL of sensitized sheep RBCs were added to each well followed by shaking and incubation at 37 °C for 20 min. The plate was read for hemolysis after centrifugation at 300 × *g* for 5 min. Samples 0%-25% hemolysis were screened as positive. From 25% to 90% the samples were screened as suspected, and 100% hemolysis was considered negative. Plus 4 results were considered an acceptable limit for control. The positive samples were tested for anticomplementry results by mixing 0.025 mL of deactivated serum with an equal volume of antigen and VBD in separate wells. In the well with VBD, 100% hemolysis was a confirmed positive result; otherwise it was anticomplementry.

Statistical analysis of results was done by t-test, in order to find the significant different between cELISA and CFT.

Results

cELISA. Serum samples from 226,923 cattle were analyzed by cELISA. The cutoff point selected for this study was 30% inhibition. Among the samples, 15,407 (6.8%) were positive, 211,494 (95%) were negative, and 13 (0.006%) were suspect (Table).

CFT. Serum samples from 211,484 cattle were analyzed by CFT. The cutoff point that discriminated positive from suspected cattle was 25% hemolysis. A total of 5105 (2.4%) samples were positive, 7193 (3.4%) were suspect, 199,186 (94%) were negative, and 274 (0.13%) were anticomplementary (Table).

Statistical analysis also showed a significant difference between cELISA and CFT.

Table. CFT and ELISA test results fo cattle anaplosmosis.

Year	Test	Samples	Positive	Suspected	Negative	Anticomplementry
2001-2005	CFT					
		211484	5105	7193	199186	274
2002-2010	c-ELIZA					
		226923	15407	13	211494	

Discussion

Tick-borne organisms, such as *Anaplasma*, are a significant cause of disease in domestic and wild animals in Australia, the USA, China, and other countries (15). Gill et al. (16) reported that in Minnesota seropositivity rates for tick borne diseases in white tailed deer, at sites with established *I. scapularis* populations, were higher.

CFT was reported as highly sensitive in recognizing induced *Anaplasma* infections in white tailed deer; however identification of *Anaplasma* species was difficult with the routinely used serological tests due to cross reactivity of titrated serum samples with the homologous and heterologous antigens (17). In our study 274 sera were anticomplementry by CFT. Similar findings were reported earlier by Howe et al. (18) in CFT on pronghorn sera. Wilson et al. (19) reported that the card agglutination, complement fixation, and indirect fluorescent antibody tests showed a stronger homologous antibody reaction when *A. marginale* antigen was tested against sera obtained from cattle infected with either *A. marginale* or *A. centrale*. Previous reports also indicated that accurate immunologic identification of persistently infected animals in areas where *A. marginale* is endemic is difficult. Antibody levels in cattle persistently infected at this low level are also difficult to detect with card agglutination, complement fixation, and indirect fluorescent antibody tests (20). Moreover, cattle in regions where *A. marginale* is endemic can be exposed to multiple rickettsial and ehrlichial agents that may induce antibodies, which are cross reactive with *A. marginale* proteins (21). Serological cross reactions among *Anaplasma* make it difficult to distinguish species and strains of this genus (9). In our study the species of *Anaplasma* were not recognized by either serological test.

In our study a cutoff of 30% was reported with cELISA. This correlates with earlier studies, which suggested 30% and 42% inhibition for this test. The test manufacturer (VMRD Inc., Pullman, WA, USA) suggested a 30% inhibition cutoff for test positives, whereas the Canadian Food Inspection Agency (CFIA) uses a 42% inhibition cutoff (22). Saliki et al. (23) reported inhibition ≥35% as a cutoff for cELISA. In order to increase the reproducibility for individual serum samples, 2 positive and 3 negative control samples were used with each plate at the University of Minnesota VDL.

It has been reported in earlier studies (24,25) that CFT had limitations due to its complexity. Using CFT for the diagnosis of anaplasmosis is complex and considerable time is needed for the standardization of CFT system components, such as complement and erythrocytes in the detection of anaplasmosis. This led the VDL at the University of Minnesota to introduce cELISA after 2002; CFT application was stopped as diagnostic test for anaplasmosis after 2005. A major advantage of cELISA was that it involved a simple procedure that was readily automated for screening large numbers of serum samples at VDL, where uniform test conditions could be maintained. Theon et al. (24) reported that CFT was a reliable serological procedure for identifying diseased animals; however, some animals infected with *A. marginale* were not detected in their study. They reported that the percentage of the animals identified as suspect by CFT (18/22) were positive by ELISA. Their findings suggested that ELISA may provide an improved test procedure for detecting infected animals in herds in which *A. marginale* infection persists. Nakamura et al. (19) reported that in the experimental infection of calves with *Anaplasma* significant antibody levels against *A. marginale* were detected by ELISA for longer periods than with CFT. Saliki et al. (23) reported that cELISA was more sensitive than CFT (24.9% versus 9.4%), mainly because CFT yielded "suspicious" or "anti-complementary" results in 10.5% of the sera and also failed to identify several vaccinated and carrier cattle that were cELISA positive. According to these researchers the apparent agreement between CFT and cELISA was 89.6%. Blouin et al. (26) also reported that sera collected from cattle for *Anaplasma marginale* were negative or suspicious by CFT, while the same sera were strongly positive by cELISA. Gonzalez et al. (27) reported that cELISA was able to detect cattle naturally infected with *A. marginale* with 99% sensitivities as compared to other serological tests, such as card agglutination and complement fixation, with reported sensitivities of 84% and 79%, respectively. The 99.5% specificity of cELISA after testing 208 sera from cattle in *Anaplasma*-free areas was reported by Molloy et al. (28); in sera from experimentally infected cattle specificity was 98.0% and 100% for *A. marginale*

and *A. centrale*, respectively. These findings suggest that the development of new serologic tests and monoclonal antibody techniques offers promise for serologically identifying *Anaplasma* isolates from diverse sources. In addition, it is apparent from previous studies that cELISA is both a suitable replacement for the CFT and the standard test for detection of the *A. marginale* antibody.

The *Anaplasma* cELISA has been approved by the US Department of Agriculture for bovines, but not for other species. The cELISA used in our investigations is based on a monoclonal antibody directed against *A. marginale* MSP5. Cross reactivity between *A. marginale* and *A. phagocytophylum* was previously reported. Dreher et al. (29) reported that the immunological cross reactivity is not restricted to MSP5, but may also include epitopes in other proteins of *A. marginale* and *A. phagocytophilum*. Sequence homologies have also been shown for the MSP4 gene of the 2 pathogens. From these findings it appears that positive results from serological *A. marginale* or *A. phagocytophilum* tests may result from infection with either of the agents or a cross reactive pathogen. This is especially important in cattle that are susceptible to both agents. Hence, a negative cELISA result suggests the absence of *A. marginale* infection or a very low *A. marginale* load. A positive result can be caused by an *A. marginale* or *A. phagocytophilum* infection. This calls for further confirmatory tests for *A. phagocytophylum*.

The use of cELISA in addition to, or as a replacement for, CFT for anaplasmosis offers many benefits. Samples received by the VDL that had deteriorated were often untestable. Results were delayed for these samples and the farmers involved incurred additional costs in cases where animals had to be re-bled. An additional validated test was necessary in these situations. From our experience at VDL, cattle samples are the samples that most frequently suffer hemolysis or anticomplementary reactions. Since it replaced CFT, cELISA had reduced the number of false positives and untestable samples. In addition, cELISA is a rapid assay, and the results can be measured objectively. The literature review in the present study also suggests further use of cELISA in epidemiologic investigations, particularly in areas where infections due to *rickettsia* are expanding through movement of infected animals into disease-free regions.

Acknowledgements

The authors would like to thank the Higher Education Commission of Pakistan for funding the postdoctoral fellowship of Aneela Z. Durrani.

References

1. Zaugg, J.L., Stiller, D., Coan, M.E., Lincoln, S.D.: Transmission of *Anaplasma marginale* by males of *Dermacentor andersoni* fed on an Idaho field infected, chronic carrier cow. Am. J. Vet. Res., 1986; 47: 2269-2271.
2. Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R.: Reorganization of the genera in the families Rickettsiaceae and *Anaplasmataceae* in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol., 2001; 51: 2145-2165.
3. Rajput, Z.I., Song-hua, H.U., Arijo, A.G., Habib, A., Khalid, M.: Comparative study of *Anaplasma* parasites in tick carrying buffaloes and cattle in Pakistan. J Zhejiang Univ. China., 2005; 11: 1057-1062.
4. Theiler, A.: Gall sickness of imported cattle and the protective inoculation against this disease. Agric. J. Union South Africa, 1912; 3: 7-46.
5. Ristic, M.: Anaplasmosis. In: Weinman, D., Ristic, M., Eds. Infectious Blood Diseases of Man and Animals, vol. 11. Academic Press, New York, USA, 1968: 473-542.
6. Osebold, J.W., Christensen, J.F., Longhurst, W.M., Rosen, M.N.: Latent. *Anaplasma marginale* infection in wild deer demonstrated by calf inoculations. Cornell Vet., 1962; 49: 97-115.
7. Kuttler, K.L.: Anaplasmosis. In: Davidson, W.R., Hayes, F.A., Nettles, V.F., Kellogg, F.E., Eds. Diseases and Parasites of White-Tailed Deer. Heritage Printers, Inc., Charlotte, North Carolina, 1987: 126-137.
8. Kuttler, K.L.: *Anaplasma* infections in wild and domestic ruminants: a review. Wildlife Disease Association. J. Wildl. Dis., 1984; 20: 12-20.

9. Bram, R.A.: Tick-borne livestock diseases and their vectors: the global problem. Ticks and tick-borne diseases, FAO Animal Production and Health Paper. World Anim. Rev. Rome, 1983; 36: 7-11.

10. Stuen, S.: *Anaplasma phagocytophilum*—the most widespread tick-borne infection in animals in Europe. Veterinary Research Communications, 2007; 31(Suppl. 1): 79-84.

11. Gale, K.R., Dimmock, C.M., Gartside, M., Leatch, G.: *Anaplasma marginale*: detection of carrier cattle by PCR. Int. J. Parasitol., 1996; 26: 1103-1109.

12. Rodgers, S.J., Welsh, R.D., Stebbins, M.E.: Seroprevalence of bovine anaplasmosis in Oklahoma from 1977 to 1991. J. Vet. Diagn. Invest., 1994; 6: 200-206.

13. Duzgun, A., Schuntner, C.A., Wright, I.G., Leatch, G., Waltisbuhl, D.J.: A sensitive ELISA technique for the diagnosis of *Anaplasma marginale* infections. Vet. Parasitol., 1988; 29:1-7.

14. Knowles, D., Torioni de Echaide, S., Palmer, G., McGuire, T., Stiller, D., McElwain, T.: Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocytes stages identifies persistently infected cattle. J. Clin. Microbiol., 1996; 34: 2225-2230.

15. Susana, T.D., Knowles, D.P., McGuire, T.C., Palmer, G.H., Suarez, C.E., McElwain, T.F.: Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein. J. Cl. Microbiol., 1998; 36: 777-782.

16. Gill, J.S., Mclean, R.G., Shriner, R.B., Johnson, R.C.: Serologic surveillance for the Lyme disease spirochete, *Borrelia burgdorferi*, in Minnesota by using white-tailed deer as sentinel animals. J. Cl. Microbiol., 1994; 24: 444-451.

17. Kuttler, K.L., Robinson, M., Franklin, T.: Serological response to *Anaplasma marginale* infection in splenectomized deer (*Odocoileus virginianus*) as measured by the complement-fixation and capillary tube agglutination tests. In Proc. Fifth Nat. Anaplasmosis Conf. Stillwater, Oklahoma, 1968; 82-88.

18. Howe, D.L., Hepworth, W.: Anaplasmosis in big game animals: tests on wild populations in Wyoming. Am. J. Vet. Res., 1965; 26: 1114-1120.

19. Wilson, A.J., Trueman, K.F., Spinks, G., McSorley, A.F.: Comparison of four serological tests in the detection of humoral antibodies to anaplasmosis in cattle. Aust. Vet. J., 1978; 54: 383-6.

20. Nakamura, Y., Shimizu, S., Minami, T., Ito, S.: Enzyme linked immunosorbent assay using solubilized antigen for detection of antibodies to *Anaplasma marginale*. Trop. Anim. Health Prod., 1989; 29: 259-266.

21. Van Vliet, A.H., Jongejan, M.F., Van Kleef, M., Van der Zeijst, B.A.M.: Molecular cloning, sequence analysis, and expression of the gene encoding the immu56nodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect. Immun., 1994; 62: 1451-14.

22. Joyce, V.D., Linfield, T.F.T., Bridges, M., Raths, D., Dargatz, D., Wagner, B., Block, J., Knoop, D., Walton, T.E.: Prevalence of antibodies to serotypes of *Bluetongue virus* and *Anaplasma marginale* in Montana feeder cattle. Can. Vet. J., 2006; 47: 692-694.

23. Saliki, J.T., Blouin, E.F., Rodgers, S.J., Kocan, K.M.: Use of tick cell culture-derived *Anaplasma marginale* antigen in a competitive ELISA for serodiagnosis of anaplasmosis. Ann. N.Y. Acad. Sci., 1998; 29: 273-281.

24. Thoen, C.O., Blackburn, B., Mills, K., Lomme, J., Hopkins, M.P.: Enzyme-linked immunosorbent assay for detecting antibodies in cattle in a herd in which anaplasmosis was diagnosed. J. Clin. Microbiol., 1980; 11: 499-502.

25. Gian, C.W., George, M.B., Lutz, H.: Detection of antibodies to *Anaplasma marginale* by an improved enzyme-linked immunosorbent assay with sodium dodecyl sulfate-disrupted antigen. J. Cli. Microbiol., 1987; 23: 633-636

26. Blouin, E.F., Saliki, J.T., Kocan, K.M., Rodgers, S.J.: Evaluation of *Anaplasma marginale* from tick cell culture as an immunogen for cattle. Ann NY Acad Sci., 1998; 29: 253-258.

27. Gonzalez, E.F., Long, R.F., Todorovic, R.A.: Comparisons of the complement-fixation, indirect fluorescent antibody and card agglutination tests for the diagnosis of bovine anaplasmosis. Am. J. Vet. Res., 1987; 39: 1538-1541.

28. Molloy, J.B., Bowles, P.M., Knowles, D.P., McElwain, T.F., Bock, R.E., Kingston, T.G., Blight, G.W., Dalgliesh, R.J.: Comparison of a competitive inhibition ELISA and the card agglutination test for detection of antibodies to *Anaplasma marginale* and *Anaplasma centrale* in cattle. Aust. Vet. J., 1999; 77: 245-9.

29. Dreher, U.M., Fuente, J.D., Lehmann, R.H., Meli, M.L., Pusterla, N., Kocan, K.M., Woldehiwet, Z., Braun, U., Regula, G., Staerk, K.D.C., Lutz1., H.: Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. Cl. Diag. Lab. Immunol., 2005; 12: 1177-1183.