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COMPARISON OF DNA PROBE TECHNIQUE AND C-ELISA FOR DETECTION OF ANAPLASMA MARGINALE IN CATTLE

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SUMMARY

Through this study , recent and modern diagnostic techniques have been used for diagnosis of cattle anaplasmosis. Out of 255 cattle that were represented by 162 foreign and 93 native breeds, blood samples were collected for separation of serum and DNA extraction using very simple and rapid guanidine thiocyanate and celite method. This investigation, throws a spot light on, the application of C-ELISA and nucleic acid hybridization using DNA labelled probe for identification of cattle persistently infected with A. marginale. The native MSP-5 is one of the outer membrane proteins (19 kDa) of A. marginale that was recognized by MAb and immue sera in C-ELISA. r-MSP-5 was used as coating antigen and the format of C-ELISA was improved for detection of antibodies against A. marginale in both acute and carrier animals. The results of C-ELISA obtained through this work showed that 15.3% of the examined cattle were positive and also identified as 16.7% and 12.9% positive reactivity in foreign and native breeds respectively. Non-radioactive msp-2 labelled probe was used in dot-blot hybridization to diagnose bovine anaplasmosis. Only 20% of the examined animals showed positive signals in dot blot hybridization, double C-ELISA/dot-blot hybridization positivity was 34 cases proved that these animals were in acute and active stage of infection however, 17 animals showed positive reactivity only with dot blot hybridization. This work is considered as the first trial for diagnosis of bovine anaplasmosis in Egypt using these recent and modern diagnostic techniques.

INTRODUCTION

Anaplasmosis is a tick-borne haemoparasitic disease of cattle, sheep, goat, buffaloes and some wild ruminants. It is known as gall sickness dis-

ease, (Theiler, 1912). The disease is caused by the rickettsia Anaplasma marginale (Theiler, 1910): Anaplasma centrale (Theiler, 1911) : Anaplrsma ovis (Lestoquard, 1924) : Anaplasma caudatum (Krier and Ristic, 1963): Anaplasma species appear as spherial, coccoid bodies of 0.2 ñ 0.4 µ in diameter in blood smear stained with Giemsa (Philip, 1956). The parasite can be transmitted biologically by ticks or mechanically by biting flies or by blood contaminated fomites like syringes or dehorning instruments (Ristic, 1980). Once the infecting organisms penetrate erythrocytes, they divide by binary fission in erythocyte membrane bound vacuole and released organisms which are designated initial bodies, infecting other erythrocytes, (Ristic et al. 1981).

Following transmission there is a prepatent period during which there is a low but increasing percentage of parasitized erythrocytes and the infected cattle are clinically normal and followed by an acute phase during which the parasitaemia increases dramatically and severe hemolytic anaemia occurs. Dramatic weight loss, abortion and death frequently occur during the acute phase. Cattle recovered from acute disease remain persistently infected with a low level parasitaemia for long periods and serve as a reservoir for transmission of the organism (Allen et al., 1981). The antigenic structure of Anaplasma marginale is represented by six major surface proteins designated MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4 and MSP-5. The results of major surface proteins analysis of A. marginale indicated that the surface proteins are targets of protective immune responses and antigenically polymorphic (Palmer et al. 1994b). Currently, diagnosis of anaplasmosis is usually applied by card agglutination, complement fixation, indirect fluorescent antibody, passive haemagglutination, capillary tube agglutination, latex haemagglutination tests and enzyme-linked immunosorbent assay. The antigen in all the currently used tests is a mixture of Anaplasma and erythrocyte stroma. Consequently, animal with antibody to erythrocytes components which developed following hemolytic disease may be falsely detected as positive using current tests, while false negatives are caused by a lack of sensitivity (Winkler et al. 1987). Research workers developed two recombinant proteins, r-MSP-3 and r-MSP-5, which are strongly and specifically recognized by cattle infected with Anaplasma marginale. Antibodies specific to r-MSP-3 are most readily detected by immunoblot assay (McGuire et al. 1991) while antibodies to MSP-5 were identified by a competitive inhibition enzyme-linked immunosorbent assay using specific MAb to rMSP-5 which has proved a great potential as a diagnostic antigen for identifying the persistently infected animals in addition to streptavidin biotin system to increase sensitivity (Palmer et al. 1994a). Recent dramatic developments in biotechnology are creating new opportunities for detecting and controlling anaplasmosis. Direct detection of infected carrier animals using the developed recombinant DNA probes and nucleic acid

hybridization in addition to polymerase chain reaction "PCR" allows us to overcome this impediments. This msp-2 probe had been developed, used to detect A. marginale in blood of infected animals and proved to be a high sensitive diagnostic tool (Palmer et al. 1994b).

This study was designed to investigate the presence of A. marginale among the Egyptian cattle, application and establishment of C-ELISA as a new technique for detection of antibodies against A. marginale in cattle sera. In addition to adopt and implate DNA hybridization technique using non-radioactive labelled DNA probe in dot blot hybridization. Finally to compare sensitivity and specificity of C-ELISA and DNA probe of Anaplasma marginale for diagnosis of cattle anaplasmosis in Egypt.

MATERIAL AND METHOD

Samples : Two hundreds fifty five venous blood samples were randomly and aseptically collected from apparently healthy mature cows and bulls over one year during the summer season, either by venipuncture or during slaughtering. These blood samples were collected into vacutainer tubes containing EDTA as anticoagulant for DNA extraction and into vacutainer tubes without anticoagulant for serum separation. These 255 blood samples referred to 162 samples collected from foreign cattle breed and 93 samples collected from native cattle breed.

Antigen: Recombinant A. marginale major surface protein-5 "r-MSP-5" was kindly obtained from Dr. A.F. Barber, Department of Veterinary Infectious Diseases, University of Florida, Gainesville, U.S.A. The recombinant bacterial lysate "E-coli XL-1 Blue" was prepared by Visser et al (1992). Anti-MAP-5 MAb "designated as ANAF16C1 by Visser et al (1992)" . Anti-species biotinylated horse anti-mouse antibody was purchased from Vector Laboratories, U.S.A.

Strept-aviding alkaline phosphatase mixture A and B: were purchased from Vector Laboratories, U.S.A.

Reference control sera : Positive control serum samples were collected from splenectomized calves experimentally inoculated with A. marginale after 70% rickettsaemia while negative control serum samples were collected from calves before inoculation.

A. marginale msp-2 probe "DNA template": A. marginale msp-2 probe was prepared according to Palmer et al. (1994b) by cloning of msp-2 gene in pCKR 11.2 plasmid and then obtained by PCR amplification between nucleotides 10 and 1226.

the msp-2 probe was 1.216 bp. Klenow enzyme labelling grade was purchased from Boehringer Mannheim GmbH.

DNA reference control samples: DNA positive control was genomic DNA of A. marginale while, DNA negative control was DNA of bovineleukocytes. Anti-digoxigenin-alkaline phosphatase fab fragments was purchased from Boehringer Mannheim GmbH. **p-Nitrophenyl phosphate :** (Sigma Chemical Co., St. Louis). L6 (lysis buffer) : 120 g guanidine thiocyanate in 100 ml of 0.1 M Tris HCl pH 6.4; 22 ml of 0.2M EDTA pH 8.0 and 2.6 ml triton x 100. L2 (washing buffer) 120 g guanidine thiocyanate in 100 ml of 0.1M tris HCl pH 6.4. Celite solution : 20% of Diamonaceous earth (celite) in 1% HCl.

Hexanucleotide mixture (10X) and; dNTP labelling mixture (10X) and Lumigen PPD were purchased from Boehringer Mannheim GmbH.

Preparation of erythrocytes for DNA extraction:

This method was carried out according to Goff et al. (1990). Briefly blood samples for DNA probe testing were collected in EDTA anticoagulant vacutainer tuber, brought to the laboratory in ice box and were washed four times with cold 1X PBS (washing buffer). After each wash, the samples were centrifuged at 3000 rpm for 15 minutes. The plasma and buffy coat were removed after each centrifugation. Erythrocytes were washed a final time with PBS containing 0.05% EDTA (PBS/EDTA), resuspended in PBS/EDTA to 50% packed cell volume in 1 ml aliquot using cryovials and then stored at -70°C.

Streptavidin-biotin based Competitive inhibition ELISA (C-ELISA):

C-ELISA protocol was carried out according to Visser et al. (1992) and Palmer et al. (1994a) using rMSP-5 as a coating antigen. Following the blocking of free antigen binding sites of ELISA coated plates with 20% skimmed milk in PBS, 100 ml of 1 : 10 cattle sera under testing and both positive and negative reference sera were added and incubated at room temperature for 30 minutes. Then 7 µl of diluted MAb (ANAF15C1) containing 5µg were added to each well and incubated for one hr, followed by 3X washing and addition of biotin-conjugated horse anti-mouse antibodies (anti-species). After 30 minutes of incubation at room temperature and 3X washing streptavidin alkaline-phosphatase mixture was added to each well for 30 minutes at room temperature. During the last 5 minutes of incubation P-nitrophenyl-phosphate was prepared in substrate buffer and 100 µl of this substrate were added to each well and incubated for 30 minutes followed by stopping of the reaction by 50 µl of 0.2 M EDTA per well . The optical density (OD) of

the developed yellow-colour was measured by reading the absorbance at 405 nm using Bio-Tek ELISA reader. The percentage of inhibition was calculated according to the following formula (Anderson, 1984).

$$\% \text{ of inhibition} = 100 - \frac{\text{OD of tested serum sample}}{\text{OD of negative serum sample}} \times 100$$

Serum samples that showed inhibition percentage of ? 40 were considered positive according to Visser et al. (1992) and Palmer et al. (1994a), who indicated that fourty percent inhibition, is represented by a value of greater than three times the standard deviation above the mean percent inhibition of 10 negative serum samples used as standards.

Guanidine thiocyanate and celite method for DNA extraction:

This method was carried out according to the current protocol of A.F. Barbet laboratory, Department of Veterinary Infectious Diseases, University of Florida, Gainesiville, U.S.A. The stabilates (washed RBC's, buffy coat removed and stored 1: 1 in PBS/EDTS) were frozen at - 70°C and thawed three times to effect erythrocyte lysis. Erythrocyte lysates were centrifuged at 14.000 rpm for 5 minutes and the supernatant was discarded to remove excess haemoglobin. The pellets were washed twice in 1 ml Tris EDTA buffer (L2) pH 8.0, inverted to mix and centrifuged at 14.000 rpm for 5 minutes. The pellets were resuspended in 900 µl of L6 Lysis buffer for membrane lysis and 60 µl celite solution to bind with DNA and were incubated at room temperature for 10 minutes. Following centrifugation the pellets were wash twice in 1 ml L2 washing buffer containing guanidine thiocyanate to assure complete lysis. Then ceutrifuged and the DNA was precipitated with 70% ethanol. The pellet was resuspended in 1 ml acetone and centrifuged then speed voc dried and resuspeuded in TE buffer, for agarose gel electrophoresis and dot botting.

Agarose gel electrophoresis: This method was carried out according to Stich et al. (1993) using 1% agarose in IX TBE buffer.

Random-primed DNA labelling msp-2 probe with digoxigenin-11-dUTP: This method was carried out according to the Genius system user's guide for filter hybridization of Boehringer Mannheim, GmbH, as follows 50 ng DNA template, 2µl Hexanucleotiede mixture (10X), 2µl dNTP labeling mixture (10X), double distilled water up to 19µl, and 1µl Klenow enzyme (100 units/ml)

Dot blot hybridization: Dot blot hybridization using DNA labelled msp-2 probe with digoxigenin-11-dUTP was carried out according to Boehringer Mannheim genius system user's guide for filter hybridization and was detected using Boeh-

ringer Mannheim chemiluminescent detection kit, GmbH, Germany.

RESULTS

Results of C-ELISA detection of antibodies to Anaplasma marginale MSP-5 in cattle sera:

A competitive inhibition enzyme-linked immunosorbent assay (C-ELISA) was developed and applied to detect antibodies against A. marginale major surface protein-5 (MSP-5) in cattle sera. C-ELISA format was based on recombinant MSP-5 protein, anti-MSP-5 MAb that inhibit and compete with tested bovine sera. Out of the examined 255 cattle sera, 162 sera were collected from foreign breed and the rest (93) were collected from native breed. The positive reactivity exhibited by both foreign and native breeds showed 27 (16.7%) and 12 (12.9%) serum samples respectively as shown in table (1). A number of 2, 9, 3, 3 and 5 serum samples inhibited the reactivity of MAb with ≥ 90, ≥80, ≥70, ≥60 and ≥50 respectively, while the majority of positive sera (17) showed percentage of inhibition ≥ 40 as tabulated in table (2). The percentages of inhibition of tested sera obteained from foreign breed were significantly higher in comparison to that obtained by using native breed sera, where 16 foreign breed serum exhibited ≥ 60% if compared to only one native breed sera that showed ≥ 60% inhibition. The majority of positivity obtained by native breed sera was ranging between ≥ 40% to ≥ 50% of inhibition (table 2). The overall percentage of positivie reactivity obtoined by CELISA was 15.3% as shown in table (1).

Results DNA dot-blot hybridization using dig-labelled msp-2 probe:

The results obtained by dot-blotting hybridization of the extracted DNA samples using the dig-labelled msp-2 probe identified 51 (20.2%) DNA samples that showed hybridization signal as shown in table (3). In addition to the genomic DNA of A. marginale (+ve control showed positive hybridization signal while the bovine leukocytes DNA (-ve control) didnít show any positive reactivity. Dig-labelled msp-2 probe identified 34 (20.1%) DNA extracted samples from 162 foreign cattle breed. Out of these 34 dot-blot hybridization positive samples, 24 samples were also positive in C-ELISA i.e. double positive for dot-blot hybridization/C-ELISA while the rest (10 samples) were dot-blot hybridization positive and C-ELISA negative. The remained 128 that failed to hybridize with the dig-labelled msp-2 probe and also showed only 3 seropositive samples as detected by C-ELISA, i.e. dot-blot hybridization negative/C-ELISA positive (table 4). Regarding to the native breed, dig-labelled msp-2 probe identified 17 (18.2%) DNA extracted samples. Out of these 17 dot-blot hybridization positive samples, 10 samples were also positive in C-

ELISA i.e. double positive for dot-blot hybridization/C-ELISA while the rest (7 samples) were dot-blot hybridization positive and C-ELISA negative. The remaining 76 that failed to hybridize with the dig-labelled msp-2 probe although only 2 seropositive samples were detected by C-ELISA i.e. dot-blot hybridization negative/C-ELISA positive as shown in table (4).

Table (1): Comparison of the results obtained by C-FLISA in both foreign and native cattle breeds sera:

Breed Results	Foreign (162)		Native (93)		Total (255)	
	No.	%	No.	%	No.	%
Positive	27	16.7	12	12.9	39	15.3
Negative	135	83.3	81	87.1	216	84.7

Table (2): Comparison of percentages of foreign and native cattle breed sera obtained by C-ELISA.

% of inhibition	Foreign		Native	
	No. of samples "162"	%	No. of samples "93"	%
≥ 90	2	1.2	-	0
≥ 80	8	4.9	1	1.1
≥70	3	1.9	-	0
≥ 60	3	1.9	-	0
≥ 50	2	1.2	3	3.2
≥ 40	9	5.6	8	8.6
<40	135	83.3	81	87.1

Table (3): Comparison of the results of dot-blot hybridization and C- ELISA

Test Breed	Dot-blot hybridization		Native (93)		Total (255)
	+ve No.	+ve %	+ve No.	+ve %	
Positive	34	20.1	27	16.7	162
Negative	17	18.2	12	12.9	93
Total number	51	20.0	39	15.3	255

Table (4): Comparison between results of dot-blot hybridization and C- ELISA of the examined cattle.

Dot-Blot/ C-ELISA Breed	+ve/ +ve	+ve/ -ve	-ve/ +ve	-ve/ -ve	Total Number
Positive	24	10	3	125	162
Negative	10	7	2	74	93
Total number	34	17	5	199	255

DISCUSSION

Early diagnosis of anaplasmosis is of critical importance to prevent spread and losses especially in case of acute disease, where death occurs in approximately one-third of untreated cattle (Alderink and Dietrich, 1981). Cattle which spontaneously recover from acute disease remain persistently infected. These animals, known as carriers, serve as reservoirs for disease transmission and allow continual outbreaks to occur to susceptible cattle (Zaugg et al. 1986). Since immediate therapy is necessary to prevent losses during acute infection, rapid accurate diagnosis of acute anaplasmosis is important. Currently acute infection is diagnosed on the basis of history, clinical signs and demonstration of intraerythrocytic rickettsia in a blood demonstration of intraerythrocytic rickettsia in a blood smear (Trueblood et al. 1991). In contrast to the microscopical identifi-

cation of intraerythrocutic rickettsia in acute infection, the chronic carriers show undetectable initial bodies (Zaugg et al. 1986). To address this problem, the MSP-5 competitive inhibition enzyme-linked immunosorbent assay was developed and improved to detect antibodies to A. marginale MSP-5 in cattle sera. The native MSP-5 is one of the outer membrane proteins (19 kDa) of A. marginale that was recognized by anti-A. marginale and anti-A. centrale immune sera in C-ELISA (Visser et al. 1992). Therefore, the presence of MSP-5 in all Anaplasma species suggest that MSP-5 is important in the Anaplasma life cycle. Also the prosence of antibody to the epitope defined by MAb in all postinfection sera indicates that this epitope is a potential diagnostic antigen for use in identifying persistently infected cattle (Visser et al. 1992).

Based on the aforementioned data, the A. marginale msp-5 gene was cloned and expressed in E. coli and the recombinant protein (rMSP-5) was used in C-ELISA as a coating antigen. The advantages of the recombinant MSP-5 used in this work were shown to overcome the non-specific and cross reactivity due to the presence of antibodies against erythrocyte material in the A. marginale antigen of experimentally infected animals (Barry et al. 1986). Such cross reaction would not be available in our assay due to the using of such rMSP-5, also batch to batch variations are negligible. This recombinant protein antigen (r-MSP-5) proved to be more specific, overcomes antigen purity problems, with exposed epitope to bind specifically with its antibody, easily reproducible in-vitro with high amount through expression from the recombinant plasmid and without need to infect the animals experimentally for antigen preparation. Moreover, the geographic conservation of the epitope recognized by MAb allows reliable use of C-ELISA to detect Anaplasma species in sera from cattle worldwide, (Palmer et al. 1994a).

C-ELISA offers a number of advantages as it requires minute amount of antigen and the results are objective and recorded automatically, thus facilitate large scale field scureening and vaccination follow up (Shkap et al. 1990). It was proved to be a simple, rapid, low cost sensitive assay. In addition, ELISA was capable to detect antibodies for more than 3 years after infection, at least 2 years longer than did a complement fixation test (Barry et al. 1986). Furthermore, the detection system of the reaction of the C-ELISA used in this study depend on the anti-mouse conjugate to detect the reacted MAb, thus the use of antibovine conjugate for the detection of reacted bovine antibody with the coated antigen is not required. This indicated that, through this assay there was a possibility for anti-A. marginale (IgM and IgG) to react with the coated protein antigen (Visser et al. 1992). In contrast to the limited detection of anti-

A. marginale IgG by anti - IgG conjugate applied by Winkler et al. (1987). The results of C-ELISA obtained in this study revealed that the positivity percentage of the examined 255 cattle sera was 15.3%. Using this technique the percentage of positive reaction could be also identified in both foreign (16.7%) and native (12.9%) breeds. Regarding to the higher positivity percentage obtained by foreign breed sera (16.7%) in relation to the native Egyptian breed percentage (12.9%) these results were expected as the anaplasmosis is endemic through out much of the world particularly the tropics and subtropics (Goff et al. 1990). These results agree to some extent and supported by those obtained by Eid, (1995) who found 19% of the Egyptian cattle showed positive reactivity using a modified C-ELISA, furthermore Eid et al. (1995) reported a higher percentage of positivity in camel sera (32.3%) using the same test . On a global scale, the higher percentage of inhibition (≥60 to ≥90) among the examined foreign breed (16%) indicating that these animals were in active subacute stage of infection during this time. In contrast, the predominant low positive percentage of inhibition obtained could explain that these animals were either in the early stage of infection or a chronic carriers, (Mass et al. 1986).

The development of nucleic acid probes and their application in diagnosing infectious diseases is becoming an attractive, alternative or supplement to conventional serologic tests (Landegren et al. 1988), especially in cases were sensitivity and/or specificity is a problem. A major concern with anaplasmosis serology is the inability to discriminate between animals with active infections and those that have been vaccinated. Thus, the ability to detect the actual organism becomes an attractive adjunct to the use of a sensitive serological test, (Goff et al. 1990). In this study the description and the development of DNA probe for detection of anaplasmosis in Egypt, in addition to the comparison between the results of C-ELISA and DNA probe were attempted. MSP-2 non-radioactive labelled probe was used in dot-blot hybridization assay in this investigation as a diagnostic tool for cattle anaplasmosis. Specificity and sensitivity experiments using heterologous (Bovine leukocytes DNA) and homologous (A. marginale genomic DNA of Florida isolate). This confirmed the results obtained previously by Palmer et al. (1994b) in this respect concerning the sensitivity and specificity of the msp-2 probe. This msp-2 probe can bind complementary to the whole copies that represent the msp-2 gene as previously proved by Palmer et al. (1994b) who indicated that the msp-2 gene is encoded by multigene family that is distributed throughout the genomic DNA of A. marginale. The msp-2 labelled probe demonstrated 100% specificity and high sensitivity by hybridization in dot blotting. The probe can detect 500-1000 infected erythrocytes in 0.5 ml of blood, which corresponds to a parasi-

taemia of 0.000025% (Eriks et al. 1989). Therefore, the probes are about 4000 times more sensitive than methods based on microscopic detection of stained erythrocytes. Moreover, the probe can be used to identify and quantitate the low, microscopically undetectable levels of parasitaemia found in cattel proven to be carriers of anaplasmosis for the least 3 years, as previously proved by Eriks et al. (1989).

The results obtained by dot blot hybridization using dig-labelled msp-2 probe were 51 (20.2%) samples showing positive signals out of the examined 255 samples. Regarding the positive percentages obtained by foreign (20.1%) and native (18.2%) cattle breeds, it seems to be nearly the same. The higher positive reactivity showed by dot blot hybridization using DNA probe than that obtained by C-ELISA may explain the sensitivity of the test as previously explained by Goff et al. (1990). The agreement of the positive results between dot blot hybridization and C-ELISA reveals positive dot blot hybridization/positive C-ELISA as represented by 24 foreign and 10 native breeds suggesting that these animals were in active subacute stage of infection, during this time, both of the parasite and antibody could be detected by dot blot hybridization and C-ELISA respectively as explained by Goff et al. (1990) and Eid (1995). The positive dot blot /negative results of C-ELISA as represented by 10 foreign and 7 native breeds samples may be attributed to low undetectable level of circulating antibody especially in early stage of infection and in chronic carrier animals as has been proved previously by Gonzalez et al. (1978) and Mass et al. (1986). Another concern of negative reactivity exhibited by C-ELISA is due to the genetic inability of the animals to produce antibody intraerythrocytic parasite will of course make initial bodies which represent the antigenic inducer away and non accessible to the immune cells. In contrase, the explanation of the positive C-ELISA/negative dot blot hybridization results as represented by 3 foreign and 2 native breeds samples could be justified by the presence of residual antibody following elemination of the parasite either spontaneously or after chemotherapy (Goff et al. 1990). Beside the development of anamnestic immune response would be expected to control the rickettsaemia levels (Kieser et al. 1990). An overview on the results obtained by dot blot hybridization (20.2%) and C-ELISA (15.3%), revealed the higher sensitivity of dot blot hybridization as expected and previously explained. These recent diagnostic tools for anaplasmosis have been established and applied for the first time in our laboratory and it should be applied for wide scale screening to detect the incidence of anaplasmosis in Egypt.

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