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SYNTHESIS AND CLONING OF cDNA OF THE EXPRESSED MSP2 GENE COPIES OF *ANAPLASMA mARGINALE*

By

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SUMMARY

Our previous data, indicated that major surface protein-2 (MSP2) gene of *A. marginale* Florida isolate is represented by a multigene family and distributed throughout the genomic DNA of *A. marginale* (Palmer et al., 1994). This results forced us to think about cloning of the expressed copies of MSP2 gene. We improved the technique of staining and separating of *A. marginale* RNA. The formaldehyde gel electrophoresis, northern blotting, hybridization using non-radiocactive labeled probe (Dig-Labeled MSP2) and chemiluminescent detection method were used. Specific MSP2 mRNA of *A. marginale* was isolated from total RNA using hybridization selection. The recovered MSP2 mRNA was used as a template for cDNA synthesis using random hexamers as a primer. cDNA synthesis was carried out for the first time for *A. marginale* cDNA library was constructed in PGEM-Z7f (+) plasmid, 5000 colonies were screened by colony hybridization using Dig-labeled MSP2 probe and chemiluminescence detection. Out of these 5000 colonies only 13 cDNA clones could hybridize specifically with Dig-labeled MSP2 probe. Restriction mapping and Southern blot hybridization of the 13 cDNA clones showed different cDNA inserts of molecular sizes ranged from 0.48 to 1.2 kbp, Sequence analysis of the 3-and 5-ends of all 13 cDNA clones were done using silver sequence "DNA sequencing system" (Promega) using T7 and Sp6 primers.

INTRODUCTION

Anaplasmosis is one of the most important haemoparasitic diseases of cattle. The disease is characterized by sever anemia caused by intra-erythrocytic infection with rickettsia *A. marginale* (Theller, 1910), abortion, dramatic weight loss, or death among infected animals. The rickettsia is transmitted either via infected ixodid ticks or mechanically or blood-contaminated surgical instruments, needles, or mouth parts of biting flies *(Rickey, 1981)*.Great economic losses due to infection greatly hinder meat and milk production. The disease remains without effective control. One approach toward vaccine development is to use a surface protein of the organisms produced and possibly delivered by recombinant methods. The protein MSP2 (for major surface protein), when prepared by affinity chromotography from infected erythrocytes, confers immunity on cattle against challenge with homologous and heterologous isolates of *A. marginale* (Palmer et al., 1988).

MSP2 gene of *A. marginale* is responsible for expression and encoding of 33-36 KD MSP2 surface protein (Palmer et al., 1988). Recent results indicated that this gene is a multigene family distributed throughout the genomic DNA

of *A. marginale* of different isolates (Palmer et al., 1994). This study aimed to 1) improve the technique of staining and electrophoresis of RAN as well as northern blotting and hybridization techniques, 2) to isolate the specific MSP2 mRNA from total RNA of *A. marginale*, 3) to give an idea shout cloning and characterization of the expressed copies of MSP2 multigene family through cDNA synthesis from mRNA of *A. marginale* to determine which copy representing and encoding for the recombinant protein that could be used as a vaccine candidate.

MATERIAL AND METHODS

MSP2 gene (original copy):

The original copy of MSP2 gene has been cloned by Dr. Pulmer (Wasington State University, Pullman), this MSP2 gene was used as a probe after labelling with Digoxigenin for screenign of cDNA library and was used to isoalte the specific mRNA of MSP2 from total RNA of A. marginale by hybridization selection.

Isolation and extractionof A. marginale RNA:

Total RNA of *A. marginale* was isolated and extracted from whole blood of infected cattle with approximately 70% parasitaemia using lithium chloride/urea mehtod after some modification according to Van der Ploeg (1982).

Improved RNA staining and electrophoresis in formaldehyde gel:

Electrophoresis of RNA was carried out according to Maniatis et al., (1982) with some modification according to Gong (1992). RNA samples preparation and gel electrophoresis were perforemed as follows: each 20 µl RNA sample contained 3 µl RNA (up to 10 µg); 2 µl of 10% formaldehyde gel running buffer (0.2 M MOPS "pH 7"; 50 mM sodium citrate; 10 mM EDTA (pH 8); 3.5 µl formaldehyde (2.2 M); 10 µl formamide (37 %); 1 µl, ethidium bromide (5 ug/ul); and 0.5 µl RNasine (Promiga, # N211/1-4). The samples were heated for 10 min, at 65 °C and then chilled on ice perior to electrophoresis. Two µl of formaldehyde gel loadind boading buffer were added. Gel preparation was done by 1% agarose in 1X MOPS running buffer contained 2.2 M formamide. The running buffer was 1X MOPS gel and running at 3-4 V/Cm.

Northen Blotting and Hybridization:

Agarose gel containing fractionated RNA was transferred to nylon membrane by capillary transfer blot using 20XSSC as a transfer buffer without any prior treatment. The blot was fixed by UV cross linker (Bio-Rod). Northern blot hybridization was carried out according to Boehringer Mannheim Genius System Guide # 101-023, using Dig-labeled MSP2 probe in a hybridization solution containing 50% formamide, 5XSSC, 0.2% SDS, 0.1% N-lauroyl sarcosine, 2 % blocking reagent, and 20 mM sodium maleat (pH 7). The hybridization was done at 42°C overnight and chemiluminescent detection was carried out using Boehringer Mannheim chemiluminescent detection Kit.

Isolation of specific MSP2 mRNA (Hybridization selection):

Specific MSP2 mRNA was isolated from total RNA of *A. marginale* by hybridization to cloned MSP2 gene that has been denaturated and immobilized on nitrocellulose filter according to Parnes et al., (1981).

Binding of MSP2 DNA to nitrocellulose filter carried out using PCR amplified MSP2 DNA with approximately 20 µg; bovine DNA loaded filter was used as a negative control; the DNA blotted

filters were fixed by UV cross linker.

Hybridization and elution of mRNA (Maniatis et al., 1982):

Total RNA of A. marginale approximately 50 µg has been used in the hybridization buffer containing 65% formamide, 20 mM PIPES (pH 6.4); 0.2% SDS; 0.4 M NaCL; and 100 µg/ml E.coli TRNA (Boehringer Mannheim). Hybridization and washing was done using wash buffer (10 mM Tris "pH 7.6", 0.15 M NaCl, lmM EDTA and 0.5 % SDS) at 65°C the elution process was done using dist. water and 100 µg/ml tRNA. Eluted mRNA was treated with RQI RNase free DNase (Promega) before being used to eliminate any contaminating DNA released from the blotted membrane. The recovered mRNA has been tested and electrophoresed in denaturing gel, Northern boltted and hybridized with digoxigenin-labeled MSP2 probe.

Synthesis and cloning of cDNA:

cDNA synthesis has been done using random hexamers as a primer and Super-Script Reverse transcriptase (RT) enzyme for synthesis of the first strand of cDNA according to GIBCO BRL Super-Script Choice system instruction manual # 8090. Second strand synthesis was done by replacement synthesis which is catalyzed by E. coli DNA polymerase-I in combination with E. coli RNase-II and E. coli DNA ligase. EcoRI (NotI) adapter was used to maximize the ligation efficiency, followed by phosphorylation of EcoRI adapted cDNA using polynucleotide kinase. Size fractionation of cDNA was carried out using column chromatography. EcoRI adapted cDNA inserts were ligated to EcoRI cut and dephosphorylated pGEM-7Zf (+) plasmid vector, of Promega. Following ligation the recombinant cDNA was used for transformation of competent E. coli cells (DH5 α and XLI Blue).

Screening of cDnA Library:

5000 colonies have been picked up and growen in LB broth in the presence of the selected antibiotic (ampicillin 50 µg/ml) in 96 microtiter plates, and screened by colony hybridization using Dig-labeled MSP2 probe and chemiluminescent detection method.

Southern blot hybridization of cDNA clones:

Recombinant cDNA were extracted by INSTA mini-prep and then digested with EcoRI enzyme, fractionated by gel electrophoresis and blotted onto nitrocellulose membrane. Hybridization was carried out according to Boehringer Mannheim Genius System # 101-023 using Dig-labeled MSP2 probe and chemiluminescent detection method.

Silver sequencing:

The 3′- and 5′ ends of the cDNA clones were sequenced using silver sequencing "DNA sequencing system" of Promega (# Q4130) utilizing T7 and SP6 as primers.

RESULTS

Electrophoresis and staining of RNA:

RNA of *A. marginale* (A. M.) as well as Trypanosoma bovis (T. b.) samples were stained with ethidium bromide (5 µg/µl) in RNA samples before being denaturated and electrophoresed in formaldehyde gel with stained RNA ladder (molecular size) as in Fig. (1.A) which shows a good resolution and staining. This result indicated that adition addition or increase of ethidium bromide in the sample prior electrophoresis was useful and did not interfer either with northern blotting or hybridization.

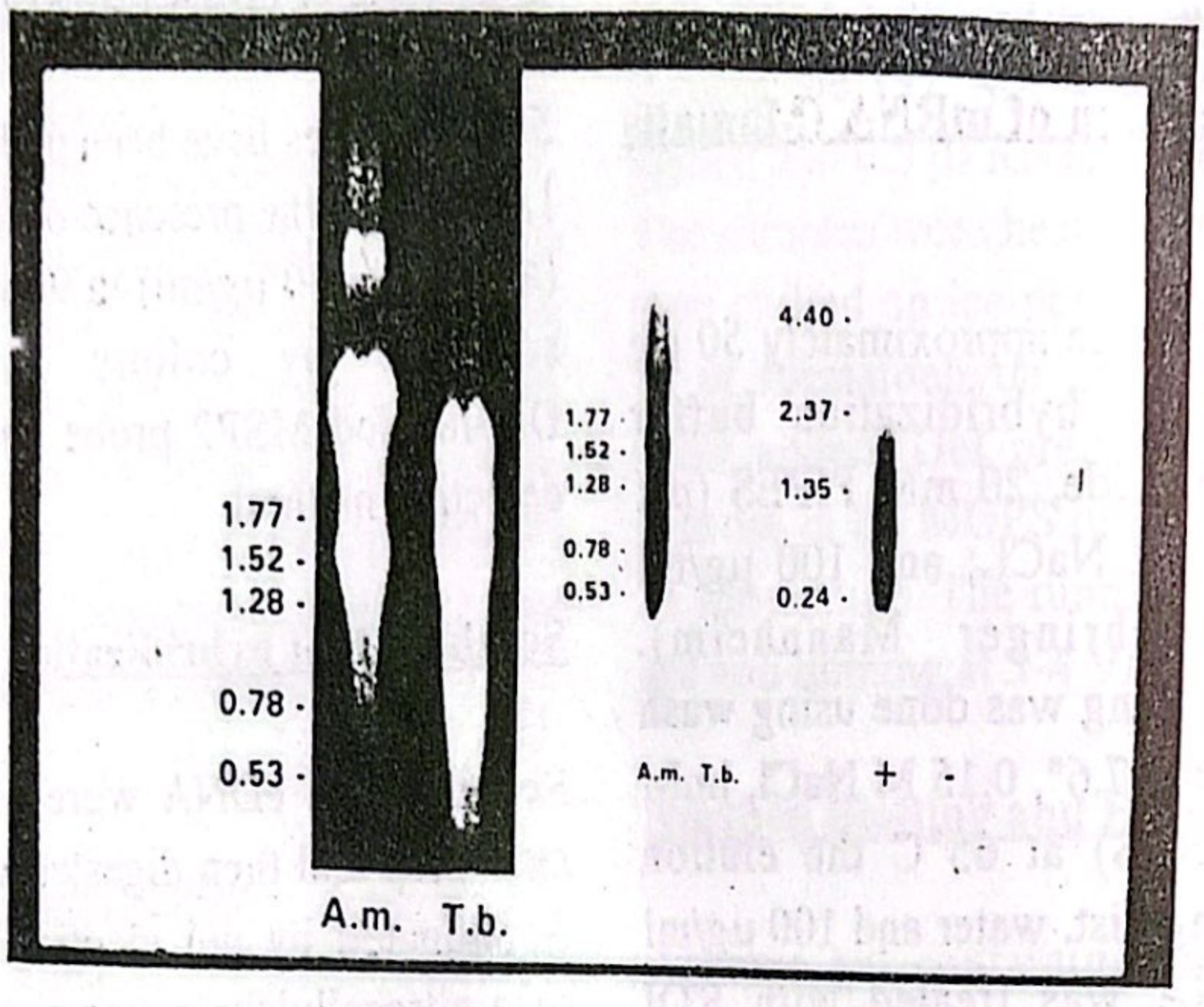


Fig. (1): Electrophoresis of Ethidium bromide stained RNA and Northern blot hybridization using Dig-labelled MSP2 probe .

Northern blot hybridization:

Electrophoresed RNA (A. m.) and (T. b.) were transfere blotted from denatured gel onto nylon membrane and hybridized with Dig-labeled-MSP2 probe and chemiluminescent detection (Fig. 1, B) indicate the specificity of the probe which hybridize only A. marginale RNA.

Testing the recovered mRNA:

Eluted mRNA from +ve (MSP2) blotted membrane and -ve (bovine DNA) blotted membrane were electrophoresed and hybridized with Dig-labeled MSP2 probe. As shown in Fig. (1, C), there is a positive reaction with the eluted mRNA of the +ve blot only, indicating that a specific mRNA specific to MSP2 gene had been recovered and in good condition that could be used as a templete for synthesis of the first strand of cDNA.

Primer selection for cDNA:

Specific oligonucleotides from the sequence of the 3 end in the flanking sequence of MSP2 gene were used as probes to hybridize with MSP2 copies in genomic DNA of *A. marginale*. The results of oligonucleotide hybridization did not show any positivity and indicated that the most conserved region of MSP2 multigene family in the middle part of the gene. Based on this result we have decided to use random hexamers as a primer for synthesis of the first strand of cDNA.

Screening of cDNA library:

5000 colonies were picked up and grown in 96 wells microtiter plates in LB broth medium containing ampicillin and screened by colony hybridization using Dig-labeled MSP2 probe and

chemiluminscence detection. The results of colony hybridization screening showed 13 positive clonies out of 5000 clones (Fig. 2).

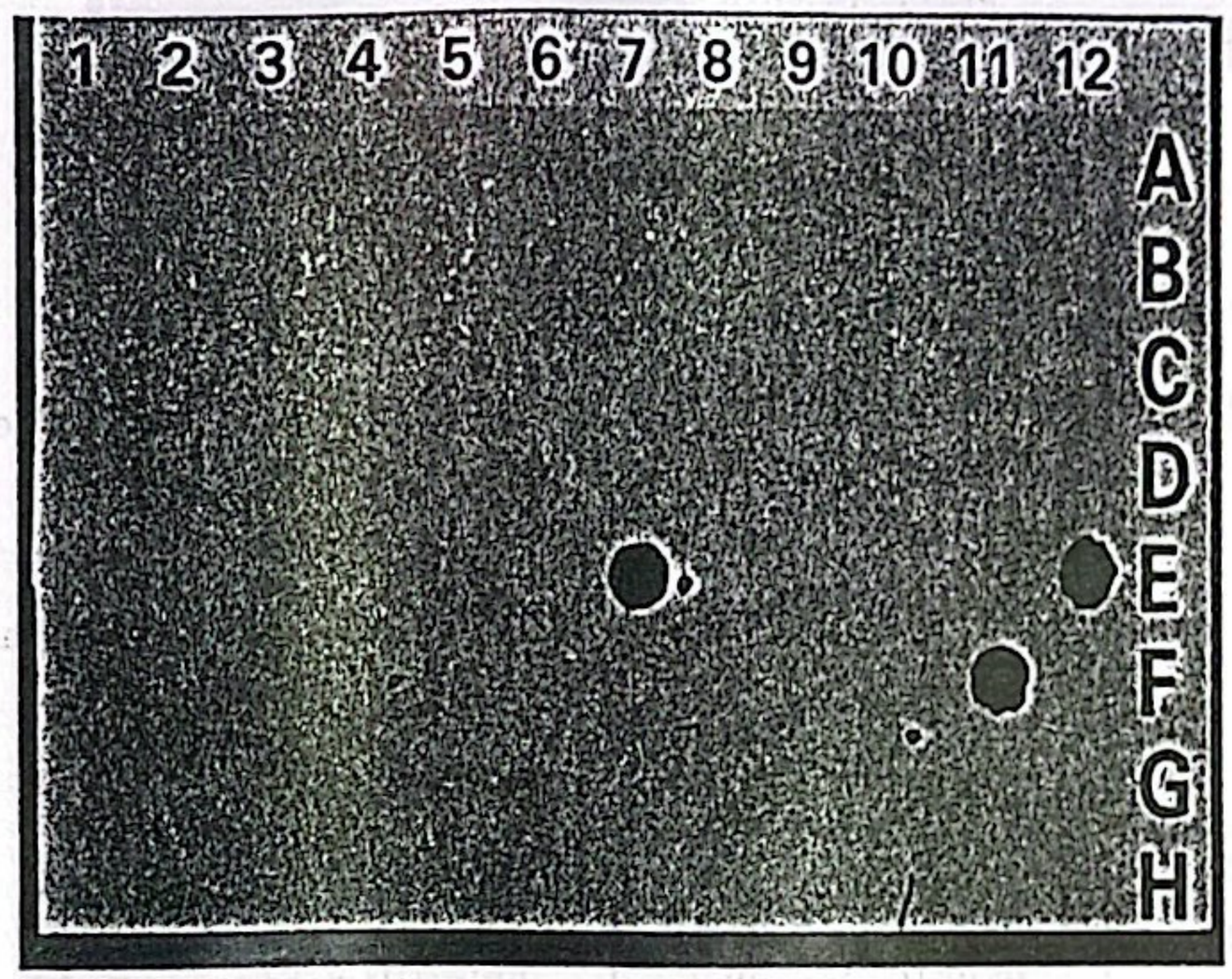


Fig. (2): Colony hybridization screening of cDNA clones .

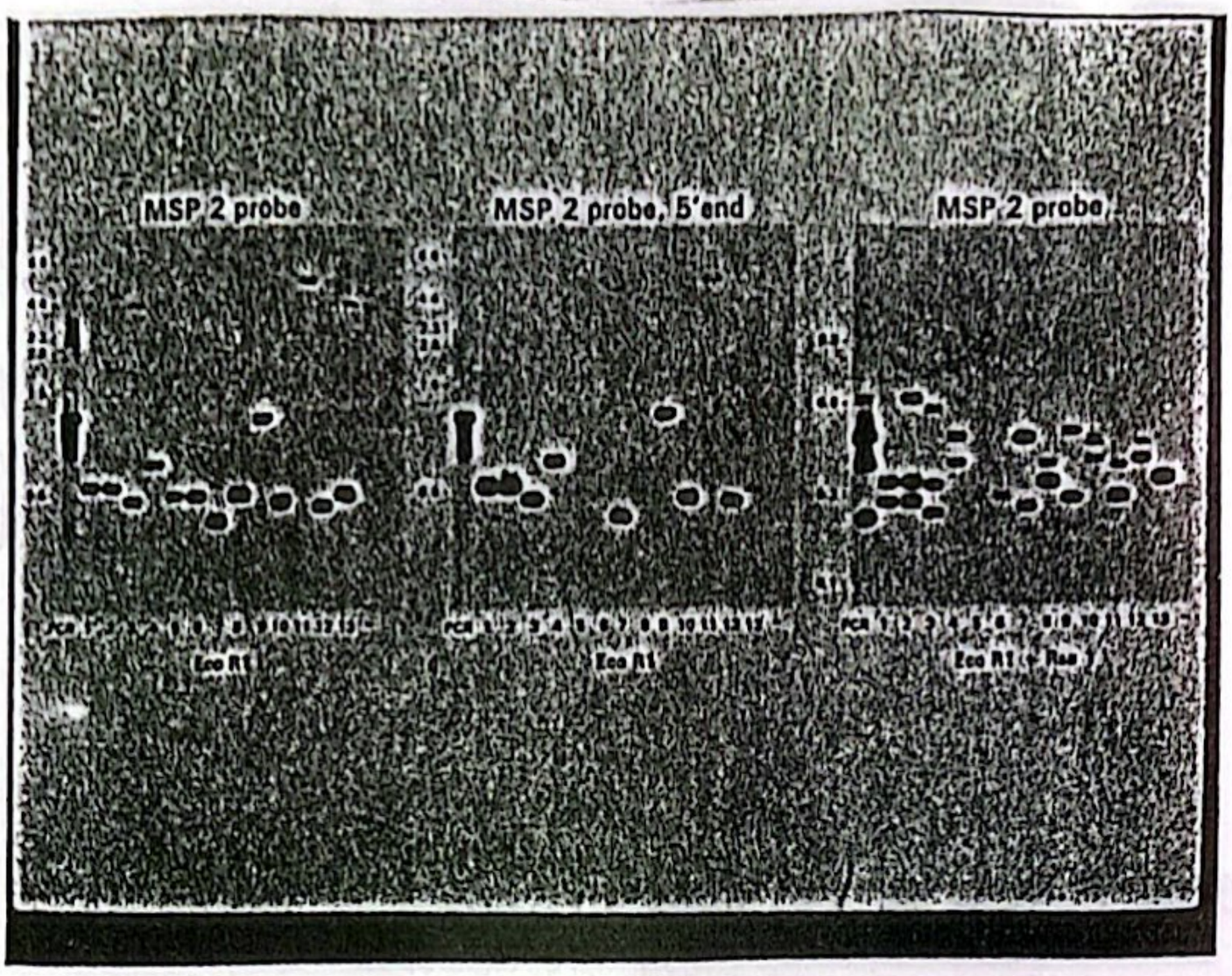
Restriction mapping and southern blot hybridization of cDNA clones:

EcoRI digested recombinant cDNA plasmid and PCR amplified MSP2 original copy as well as empty plasmid were fractionated and blotted to nylon membrane followed by hybridization with Dig-labeled probe. The results tabulated in Table (1) and Fig. (3. A) showed cDNA inserts of different molecular size ranged from 0.48-1.2 kpb. The exsistence of 5 ends were screened in all cDNA clones using Dig-labeled MSP2 probe 5 end. The results indicated that 5 end was exsist in all cDNA insert except clones No. 5 and 6 as shown in table (1) and Fig. (3, B).

Internal fragments of all cDNA clones and the original MSP2 copy were analysed after double digestion with EcoRI and RsaI restriction enzymes. The results in Fig. 3.C and Fig. 4 show similarity of the excepted size between the original whole MSP2 copy and some cDNA clones specially colnes # 4, 7, 8, 9, 10 and 13.

Sequencing analysis:

Prelimminary DNA sequencing of the ends of all 13 cDNA clones have been done using silver sequence. The results revealed that cDNA clones covered the 5- 1000 base pairs encoding MSP2 as well as approximately 800 base pairs 5 to the coding region. There were many regions of overlaping between the different cDNA clones.



3A 3B 3C

Fig. (3): Restriction maping analysis of cDNA clones, and Southern blot hybridization with Dig-labelled MSP_2 probes .

Table (1): Molecular size of cDNA inserts and exsistence of 5'end in cDNA clones .

Clone #	Molecular size (kbp) with MSP2 probe hybridization	MSP2 5'end probe hybridization
Original copy	1.2	+
1-	0.70	+
2-	0.70	+
3-	0.60	+
4-	0.86	+
5-	0.62	-
6-	0.62	-
7-	0.48	+
8-	0.62	+
9-	1.20	+
10-	0.62	+
11-	0.70	+
12-	0.60	+
13-	0.60	+

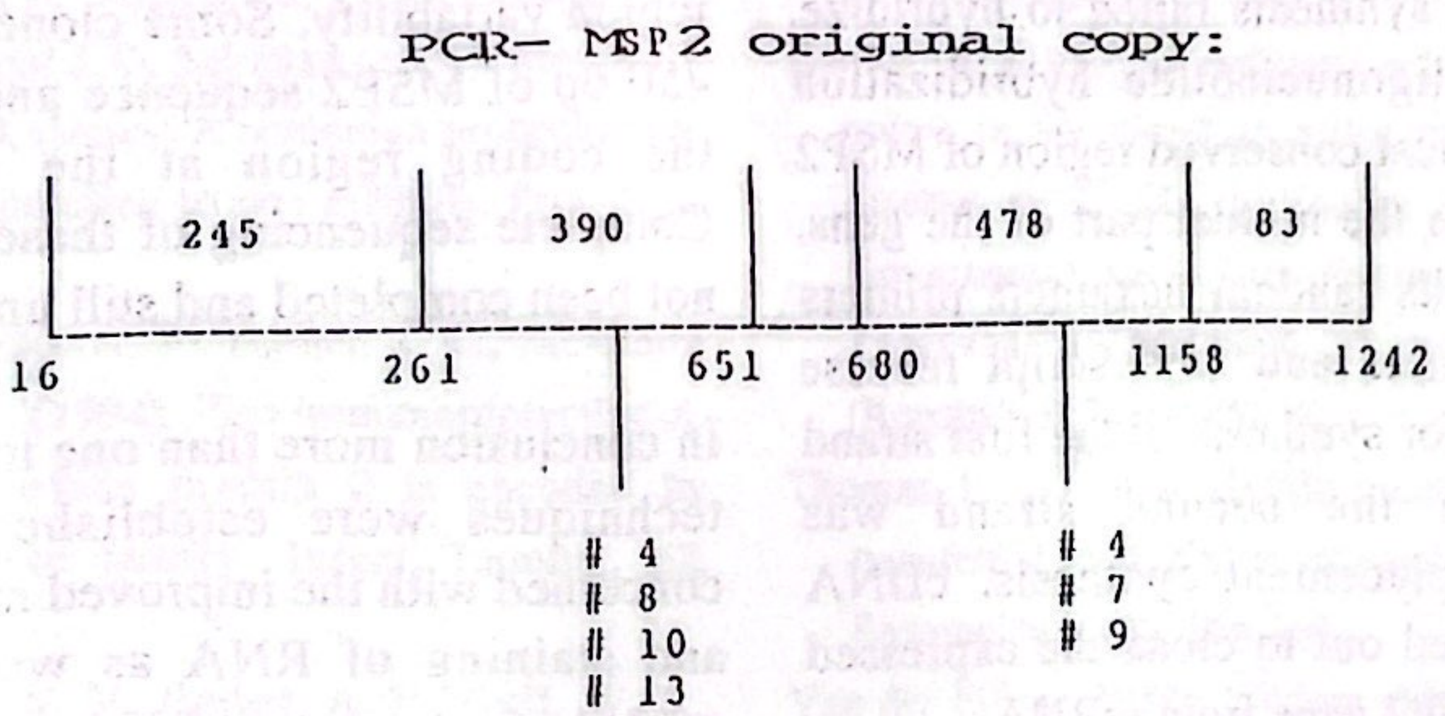


Fig. (4). Expected internal fragments from whole MSP_2 copy and cDNA clones after RsaI and EcoRI double digestion .

DISCUSSION

In general, working with RNA need special care and precaution due to the presence of ribonucleases everywhere and the shorter half time of RNA. An improved technique for staining and electrophoresis of RNA in denaturing gel was developed. Although there are different methods for staining and electrophoresis of RNA e. g. glyoxal gel electrophoresis. and urea/polyacrylamide gel electrophoresis, the application of modified formaldehyde gel electrophoresis showed a good staining resolation (Fig. 1A) without background. Our data were supported with the results obtained by others (Gong. 1992); Kroczek, 1989 and Rosen et al., 1990) they have reported that addition of ethidium bromide in the sample prior to electrophoresis is convenient and sensitive way to stain RNA samples and keep back ground fluroescence low. In contrast to, Ogretman, et al., (1993)who indicatd that addition of high concentration of ethidum briomide prior electrophoresis caused significant alteration in the RNA mobilities and decreased hybridization capacity. While optimal addition of ethidium bromide in the sample resulted in the best combination of staining sensitivity and absence of alteration of RNA mobility and efficiency of hybridization. In contrast, to those results staining of RNA in gel has been reported to decrease the efficiency of transfer to nitrocellulose membrane (Thomas, 1983).

Isolation of specific MSP2 mRNA from total RNA of prokaryotic *A. marginale* was performed using hybridization selection method and the recovered mRNA was tested (Fig. lC) with non-radioactive labeled MSP2 probe and chemiluminescent detection which has been also established durign this work, the results showed healthy isoalted mRNA which could be used as a

templete for cDNA synthesis.

Some MSP2-specific oligonucleotides from 3´ end in the flanking sequence of MSP2 were used as primers for cDNA synthesis failed to hybridize. This results of oligonucleotide hybridization indicated that the most conserved region of MSP2 multigene family in the middel part of the gens. Based on this results random hexamers primers were used as primers and superscript reverse transcriptase (RT) for synthesis of the first strand of cDNA, while the second strand was synthesised by replacement synthesis. cDNA synthesis was carried out to clone the expressed coyp (copies) of MSP2 gene from mRNA.

The data presented in this paper represent the first mRNA analysis of genes encoding in prokaryotic *A. marginale*. Screening of an *A. marginale* cDNA library with colony hybridization using non-radioactive labeled MSP2 probe catched 13 positive cDNA clones (Fig. 2) out of 5000 colonies. The molecular size of cDNA inserts was detected by Southern blot hybridization which revealed different size of cDNA inserts ranged from 0.48-1.2 kbp. (Table 1 and Fig. 3A). Further analysis of these 13 cDNA clones using Dig-labeled MSP2 probe 5 end indicated the existing of 5-end of the MSP2 gene in all cDNA inserts except 2 clones (# 5 and 6) as shown in Fig. 3B and Table (1). Restriction mapping of cDNA colnes using restriction endonucleases EcoRI and RsaI and Southern blot hybridization using Dig- labeled MSP2 probe showed to some extent great similarity of the internal fragments between the original MSP2 whole probe and most cDNA inserts specially the 390 and 478 bp pieces (Fig. 4), this means that these cDNA inserts specially synthesised from the specific mRNA of MSP2 gene. Preliminary sequence and analyses of the 3´- and 5´- ends of all 13 cDNA clones were analysed using T7 and Sp6 primers. The results indicated that cDNA clones covered the 5´-1000 base pairs encoding MSP2 as well as approximately 800 base pairs 5 to the coding region. There were many regions of these cDNA clones should allow an estimate of the minimum number of different expressed MSP2 gene and the region variability. Some clones (# 9) begins at -# 450 bp of MSP2 sequence and continues outside the coding region at the N- terminal end. Complete sequencing of these cDNA clones has not been completed and still under analysis.

In conclusion more than one molecular biological techniques were established specially these concerned with the improved methods for running and staining of RNA as well as isolation of mRNA from total RNA of prokaryotic *A. marginale* and non-radioactive labeling probe and hybridization techniques. The main goal of the work presented here was to develope a method for synthesis and cloning of cDNA form RNA of *A. marginale*, the positive cDNA clones could help us to clone the expressed copies of MSP2 gene as it is difficult to clone it from the genomic DNA of *A. marginale* due to the distribution of the gene throughout the genomic DNA, which could be use as a vaccine condidate for anaplasmosis.

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