

Original Article

Molecular Diagnostic of *Anaplasma marginale* in Carrier Cattle

Vahid Noaman, *Parviz Shayan, Narges Amininia

Center for Ticks and Tick-borne Diseases, Faculty of Veterinary Medicine,
University of Tehran, Iran

(Received 6 Sep 2008; accepted 25 Jan 2009)

Abstract:

Background: Anaplasmosis belongs to the complex of several tick-borne diseases and can cause diseases in the livestock with high economical losses. Cattle that recover from acute infection become carriers and the parasite can persist most probably for the lifetime in the blood. The aim of the present study was the determination of the persistently infected cattle in a region of Iran with the previous history of acute anaplasmosis.

Methods: One hundred and fifty blood samples and corresponding blood smears of cattle without any signs of diseases were prepared from a region in Isfahan/ Iran with the previous history of acute anaplasmosis from March 2007 to July 2007 for cross sectional study of carriers of *Anaplasma*. The blood smears were first screened by Giemsa staining, the extracted DNA from blood cells were analyzed by *Anaplasma marginale* specific nested PCR, and PCR-RFLP using primers derived from 16S rRNA gene and restriction endonuclease Bst1107 I.

Results: *Anaplasma* like structures could be identified in the limited amount of erythrocytes of 75 blood smears. In these samples, the percentage of erythrocytes harboring *Anaplasma* like structures varied from 10⁻³% to 10⁻²%. Nested-PCR and PCR-RFLP analysis showed 58 *A. marginale* positive cases within 75 *Anaplasma* suspected blood samples. In 150 total blood samples, 50% were *A. marginale* positive.

Conclusion: Our results revealed that the traditional Giemsa staining method is not applicable for the determination of the persistently infected cattle. In addition, the results showed that the carrier animals must be widespread in the *Anaplasma* endemic areas in Iran.

Keywords: *Anaplasma marginale*, Cattle, Iran, Carrier, Nested PCR, PCR-RFLP

Introduction

Species of the genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular etiological agents of tick borne diseases of mammalian hosts (1), includes the causative agents of anaplasmosis of ruminants. Of these erythrocytic *Anaplasma* spp., three species, two infecting cattle (*A. marginale* and *A. centrale*) and one in sheep and goats (*A. ovis*) were well-recognized (2- 4).

Acute anaplasmosis, caused by *A. marginale*, is one of the most important diseases of ruminants worldwide, causing significant economic losses in the tropical and subtropical areas (5, 6).

The disease is characterized by a progressive hemolytic anemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of the infected cattle (7). Diagnosis of bovine anaplasmosis performed rou-

* Corresponding Author: Email: pshayan@ut.ac.ir

tinely by Giemsa-staining of blood smear of clinically suspected animals in Iran. Giemsa-stained blood smears can be indeed used as a suitable method to detect *Anaplasma* in the animals clinically suspected for acute diseases, but it is not applicable for the determination of pre-symptomatic and carrier animals (8). It seems that the cattle recovered from acute anaplasmosis function as long-term or lifetime carrier (9). Eriks et al. (10) reported that cyclical levels of *Rickettsia* in persistently infected cattle fluctuate between $10^{2.5}$ and 10^7 infected erythrocytes/ml, with the lowest levels lasting approximately 5–8 days of every 5–6-week cycle. Since the persistently infected cattle can serve as a reservoir for the spread of *A. marginale*, they will be important for both herd health management and movement of animals into and out of the endemic areas (11). Gale et al. (12) showed that only levels of 10^6 infected erythrocytes per ml could be detected by Giemsa staining. Therefore, several serological tests have been established. Unfortunately, because of antigen cross reactivity, these tests do not discriminate between different *Anaplasma* species (11, 13, 14). Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify *A. marginale* DNA (8,15-17). In Iran, anaplasmosis are diagnosed based up on traditional morphological characteristics of Giemsa-stained blood smears, which is not surely applicable for the identifying of the carrier animals.

The aim of the present study was the determination of the persistently infected cattle in a region of Iran with the previous history of acute anaplasmosis.

Material and Methods

Collection of blood Samples

From March 2007 to July 2007, 30 farms in Isfahan Province, central part of Iran, were se-

lected for the study based on their history of outbreak of bovine anaplasmosis. Blood samples were collected from jugular vein of 150 Friesian and crossbred cattle ranging between 1 and 9 years. Five hundred micro liters of each collected blood samples was fixed with 1 ml 96% ethanol in 1.5 ml sterile eppendorf tubes. Additionally, two thin blood smears were prepared immediately after each blood collections. The blood smears were air dried, fixed in methanol, stained with Giemsa and examined microscopically for the presence of *A. marginale* in the erythrocytes. All smears carefully examined to estimate the Percent Parasitized Erythrocytes (PPE) as described by Coetzee et al. (18). To determine the carrier status of cattle, DNA was extracted from the whole blood and examined using specific primers derived from *Anaplasma* 16S rRNA gene.

DNA extraction

DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, ca. 5 mm³ big pieces of fixed blood samples was first air dried and subsequently lysed in 180 µl lysis buffer and the proteins were degraded with 20 µl proteinase K for 10 min at 55°C. After addition of 360 µl Binding buffer and incubation for 10 min at 70°C, 270 µl ethanol (96%) was added to the solution and after vortexing, the complete volume was transferred to the MBST-column. The MBST-column was first centrifuged, and then washed twice with 500 µl washing-buffer. Finally, DNA was eluted from the carrier using 100 µl Elution buffer. The amount of extracted DNA and its purity was measured by OD²⁶⁰ and the ratio of OD²⁶⁰ to OD²⁸⁰ respectively. In addition, the extracted DNA was analyzed on agarose gel before use.

Polymerase chain reaction and semi-nested PCR

Approximately 100 to 500 ng DNA was used for the PCR analysis. The PCR was performed in 100 µl total volume including one time PCR

buffer, 2.5 U Taq Polymerase (Cinnagen, Iran), 2 µl of each primer (P1/P2, 20 µM, Cinnagen), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermentas) and 1.5 mM MgCl₂ in automated Thermocycler (MWG, Germany) with the following program: 5 min incubation at 95°C to denature double strand DNA, 35-38 cycles of 45 s at 94°C (denaturing step), 45 s at 56°C (annealing step) and 45 s, at 72°C (extension step). Finally, PCR was completed with the additional extension step for 10 min. The PCR products were analyzed on 1.8% agarose gel in 0.5 times TBE buffer and visualized using ethidium bromide and UV-eluminator. To control the specificity of the PCR products for the 16S rRNA gene of *Anaplasma spp.*, nested PCR technique was used, in which the additional primers (P3/P4) from the same gene were designed upstream from forward primer (P1) and downstream from reverse primer (P2) respectively. For the determination of *A. marginale* an additional primer (P5) was designed from the hyper variable region of the 16S rRNA gene and the specificity was determined using primers P1/P5 by semi-nested PCR. Nested and semi-nested PCR were performed with the PCR product isolated from agarose gel using the MBST-Kit according to the manufacturer's instructions. Briefly, the DNA bands were cut from the gel under UV control and dissolved in the binding buffer at 60°C. The dissolved agarose was transferred into the MBST-column. After washing, the bound DNA was eluted with 30 µl TE-buffer. One to five micro liter of the eluted DNA was amplified with the primers P3/P4 or P1/P5 separately. In addition, nested and semi-nested PCR was performed directly with one µl PCR product as well. The primers are listed in the Table 1.

PCR product purification

PCR product was purified from the salts and proteins using PCR purification kit (MBST, Iran). Briefly, 200 µl binding buffer was added to 100 µl PCR product solution. After adding of 150 µl ethanol (96%) to the sample, the mixture

was transferred into the column. The column was washed twice with washing buffer and PCR product was eluted from the column using 100 µl elution buffer.

PCR-RFLP

The extracted DNA from blood samples was amplified using P1/P4 primers, resulted a PCR product of 577 bp for all *Anaplasma spp.* The PCR products were purified from enzyme and salts using PCR-product purification kit (MBST). Ten µl of purified PCR product was then cut with 0.1 µl restriction endonuclease Bst 1107 I (Roche, Germany, 10U/ µl) in 2.5 µl 10 x corresponding buffer and 12.5 µl H₂O for 1 h by 37°C. As control 10 µl PCR products was treated with 2.5 µl 10 x corresponding buffer and 12.5 µl H₂O without adding of enzyme.

Results

Analysis of blood smears

Microscopic examination revealed that 75(50%) blood smears out of 150 had *Anaplasma* like structures (ALS). All smears carefully were examined to estimate the Percent Parasitized Erythrocytes (PPE). In these samples, the percentage of erythrocytes harboring ALS varied from 10⁻³% to 10⁻²% (Fig. 1).

Analysis of blood samples by Nested-PCR and PCR-RFLP

The DNA was extracted from 150 blood samples. The purity of the DNA probes was measured and the ration of OD²⁶⁰ to OD²⁸⁰ was between 1.7 and 1.9. The amplification of the DNA probes isolated from 75 blood samples, in which ALS were detected showed an expected PCR product of 781 bp in only 58 samples (approximately 77%). The nucleotide sequence of 16S rRNA gene is much conserved in *Anaplasma spp.* and the primers P1/P2 can amplify the corresponding gene in *A. marginale*, *A. centrale*, *A. bovis*, *A. phagocytophilum* and

A. ovis. Twenty three percent of these samples were negative. PCR analysis of the DNA isolated from the rest 75 blood samples, in which no *Anaplasma* like structures were detected revealed the same expected PCR product in 17 DNA probes. Seventy seven percent of these 75 blood samples were *Anaplasma* negative. To confirm that the PCR products were *Anaplasma* spp. specific, the PCR products were amplified with the primers P3/P4, which were designed from the region flanked by the primers P1/P2. All PCR products could be amplified with the abovementioned primers (P3/P4), which denoted that the first PCR product belongs to the 16S rRNA gene of *Anaplasma* spp. The amplified nested PCR product had an expected PCR product with 543 nucleotides in length (Fig. 2B). The 16S rRNA gene of *Anaplasma* spp has a small hyper variable region; its nucleotide sequence has been used for the differentiation of *Anaplasma* spp. from each other. Therefore, another control experiment was performed using primer (P5) designed from the nucleotide sequences of *A. marginale* small hyper variable region. Amplification of all 75 PCR products with primers P1/P5 resulted in the semi nested PCR product of 120 bp (Fig. 2C). Since the nucleotide sequence of primer P5 has a high ho-

mology to the corresponding sequences in *A. centrale* published under accession no. AF414868 and AF414869 and *A. ovis* published under accession no. AF414870 and AF309865, the certain differentiation of these three species from each other was not given. Therefore, for the determination of the specificity of the PCR products for *A. marginale*, PCR-RFLP method was used. For this aim, the DNA was first amplified using P1/P2 primers. After purification of this PCR product from the agarose gel, the extracted DNA was amplified using P1/P4 primers. Then the later PCR product was purified and cut with the restriction endonuclease Bst1107I. The restriction endonuclease Bst1107I recognizes the sequence (GTATAC) in corresponding PCR product of *A. marginale* and cut it in the position 68, whereas the used restriction enzyme cannot cut the corresponding PCR product of *A. ovis* (GTACGC) or *A. centrale* (GTACGC).

Analysis of all 75 *Anaplasma* positive PCR products with the restriction endonuclease Bst1107I showed that all PCR products could be cut in two expected DNA fragments with 509 bp and 68 bp in length respectively (Fig. 2D).

Table1: *Anaplasma* spp. specific primers

PCR-product	positions	Nucleotid sequences	Accession No. in Gen-Bank	Primer
781bp	1-20	5` agagtttgatcctggetcag 3`	M60313	P1
	781-762	5` agcactcatcgtttacageg 3`	M60313	P2
543bp	35-56	5'gcaagcttaacacatgcaagtc3'	M60313	P3
	577-558	5'gttaagccctggtatttcac3'	M60313	P4
120bp	120-71	5`ctatgcattactcaccgctctgccac taaccata-cacgcagcaagctgcg 3`	M60313	P5



Fig. 1: Blood smear was analyzed by Giemsa staining *Anaplasma* like structure is to detect in one of the erythrocytes (arrow)

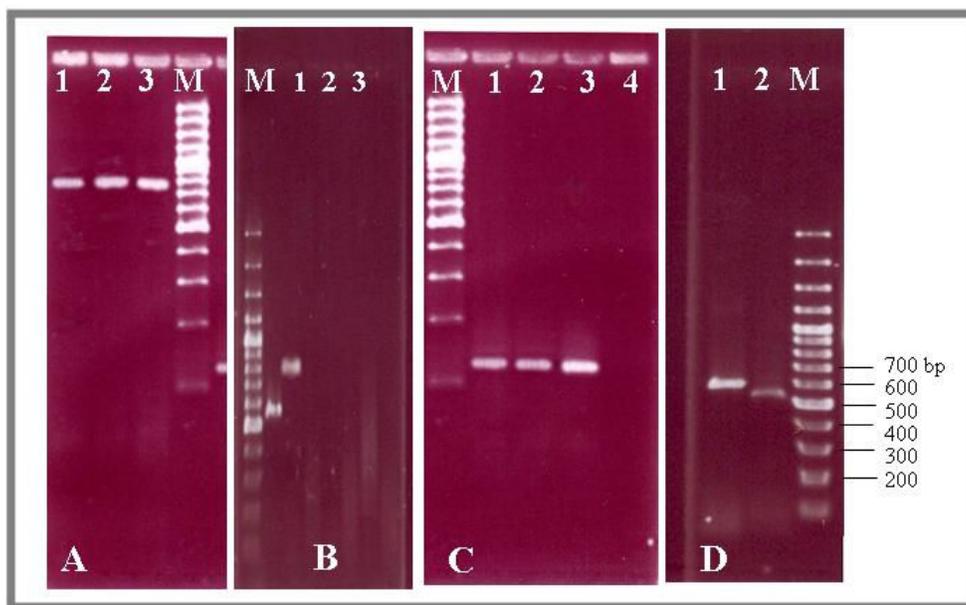


Fig. 2: DNA isolated from blood was analysed by PCR and PCR-RFLP. **A:** DNA from 3 cattle was amplified with primer P1/P2 resulting in PCR product of 781bp in length. **B:** The PCR product amplified using P1/P2 (781bp lane 2) was re-amplified with P3/P4 resulting in PCR product of 544(lane 1). Lane 3 is negative control. **C:** PCR product of 781bp was amplified by primers P1/P5 resulting in PCR product of expected 120bp in length. **D:** PCR product of 577bp (P1/P4) lane 1 was cut with restriction endonuclease BST1107I resulting in DNA fragment of 509bp (lane 2)

Discussion

Anaplasmosis is an important disease caused high economical losses in cattle industry in Iran. It is believed that *Anaplasma* can persist in cattle recovered from acute anaplasmosis (9) and can function as risk for health management in cattle industry. To determine the persistently infected cattle, blood samples from 150 cattle housing in region of province Isfahan (Iran) with the history of anaplasmosis were analyzed. Since, diagnosis of *Anaplasma* infection is detected by traditional Giemsa staining method in Iran; we have analyzed the blood samples by Giemsa staining and by PCR techniques, to have the possibility to compare the detection methods with each other.

Our results showed that *Anaplasma* like structures could be detected in erythrocytes of 50 percent of the total blood samples. The percentage of erythrocytes harboring *Anaplasma* like structures varied from $10^{-3}\%$ to $10^{-2}\%$. Our results were comparable to the results of Eriks et al. (10). Eriks et al. (10) reported that cyclical levels of *Rickettsia* in persistently infected cattle fluctuate between $10^{2.5}$ and 10^7 infected erythrocytes/ml. Due to the very low amount of the *Anaplasma* infected erythrocytes in carrier cattle, and due to the difficulty in differentiation between *Anaplasma* organisms and structures like Heinz bodies, Howell-Jolly bodies or staining artifacts, which often seen in Giemsa stained blood smears, DNA from corresponding blood samples were analyzed by PCR. PCR, nested PCR and PCR-RFLP analysis showed that 75 out of the total 150 blood samples were *A. marginale* positive. Interestingly, *Anaplasma* like structures could be detected in erythrocytes of 75 blood samples by Giemsa staining, within which only 58 blood samples were *A. marginale* positive by nested PCR and PCR-RFLP. Our results suggest that the structures recognized in erythrocytes of 17 PCR negative blood samples, could be Heinz bodies, Howell-Jolly bodies or staining artifacts. This means, due to the very low amount ($10^{-2}\%$ – $10^{-3}\%$) of in-

fectured erythrocyte in the examined carrier cattle, it is very difficult to determine the *Anaplasma* organisms by simple Giemsa staining, which is performed routinely in the laboratories in Iran. This observation was reported also by Torioni et al. (19), Bradway et al. (11) and Carelli et al. (8). Carelli et al. (8) presented the real time PCR assay for detection and quantification of rickettsemia in carrier, pre-symptomatic and symptomatic cattle. Although compared to the classical PCR, the real time PCR has some advantages such as shorter processing time and lower contamination risks, but requires expensive equipment and cannot be used now for determination of anaplasmosis in Iran.

The limited amount of infected erythrocytes can be also successfully ascertained by nested-PCR and PCR-RFLP. The present study is the first molecular based report for the existence of a remarkable number of carrier cattle in Iran. These carrier cattle can serve most probably as the reservoir of infection for vector ticks. Furthermore, the carrier status of cattle can function under severe nutritional or climatic stress for the clinical relapse. Control and management of livestock health could be understood as the two sides of a gold coin for a successful and healthy economy in stock farming. Here, the control of tick-borne diseases plays a prominent role. One of the most important diseases in cattle farms is the infection with *Anaplasma* organisms, which cause annually high economical losses in Iran. Furthermore, reviews of tick-borne diseases have been increasingly recognized worldwide as highlighting this animal health problem.

Conclusively, our results showed that nested PCR and PCR-RFLP based on 16S rRNA gene could be used as a safe method for the identifying of *A. marginale* carrier cattle. In addition, the *Anaplasma* carrier cattle must be widespread in the *Anaplasma* endemic areas in Iran,

which must be considered in the future Iranian animal health programs.

Acknowledgements

This work was supported by Grant no. 7502016/6/2 of University of Tehran and Investigating Group Molecular Biological System Transfer (MBST) in Iran.

References

1. Dumler J, Barbet A, Bekker C, Dasch G, Palmer G, Ray S, Rikihisa Y, Rurangirwa F. Reorganization of 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 HGE agent as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol*. 2001; 51, 2145–2165.
2. Lestoquard, F. Deuxieme note sur les piroplasmoses du mouton en Algerie. L'Anaplasmose: *Anaplasma ovis* nov. sp. *Bull Soc Path Exot*. 1924;17,784–787.
3. Theiler A. *Anaplasma marginale* (gen. spec. nov.). The marginale points in the blood of cattle suffering from a specific disease. In: Theiler, A. (Ed.), Report of the Government Veterinary Bacteriologist, in Transvaal. Department of Agriculture. 1910; 1908–1909.
4. Theiler A. Further investigations into anaplasmosis of South African cattle. In: First Report of the Director of Veterinary Research, Department of Agriculture of the Union of South Africa. 1911;7–46.
5. Kocan KM, Blouin EF, Barbet AF. Anaplasmosis control. Past, present, and future. *Ann N Y Acad Sci*. 2000;916, 501–509.
6. Kocan KM, de la Fuente J, Guglielmone AA, Melendez RD. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev*. 2003; 16, 698–712.
7. Palmer GH. *Anaplasma* vaccines. In: Wright IG (Ed.), *Veterinary Protozoan and Hemoparasite Vaccines*. USA: CRC Press, Boca Raton; 1989; 1–29.
8. Carelli G, Decaro N, Lorusso, A, Elia G, Lorusso E, Mari V, Ceci L, Buonavoglia C. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet Microbiol*. 2007; 124,107-114.
9. Eriks IS, Palmer GH, McGuire TC. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J Clin Microbiol*. 1989;27, 279–284.
10. Eriks IS, Stiller D, Palmer GH. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. *J Clin Microbiol*. 1993; 31, 2091–2096.
11. Bradway DS, Torioni de Echaide S, Knowles DP, Hennager SG, McElwain TF. Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale*. *J Vet Diagn Invest*. 2001;13(1), 79-81.
12. Gale KR, Dimmock CM, Gartside M, Leatch G. *Anaplasma marginale*: detection of carrier cattle by PCR. *Int J Parasitol*. 1996;26, 1103–1109.
13. de la Fuente J, Lew A, Lutz H, Meli M, Hofmann-Lehmann R, Shkap V, Molad T, Mangold A, Almazan C, Naranjo V, Gortazar C, Torina A, Caracappa S, Garcia-Perez A, Barral M, Oporoto B, Ceci L, Carelli G, Blouin E, Kocan KM.

- Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev.* 2005;6, 75–89.
14. Dreher UM, Hofmann-Lehmann R, Meli ML, Regula G, Cagienard AY, Stark KDC, Doherr MG, Filli F, Hassig M, Braun U, Kocan KM, Lutz H. Seroprevalence of anaplasmosis among cattle in Switzerland in 1998 and 2003. No evidence of an emerging disease. *Vet Microbiol.* 2005;107, 71-79.
 15. Bekker CP, de Vos A, Taoufik A, Sparagano OA, Jongejan F. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridisation. *Vet Microbiol.* 2002;89, 223–238.
 16. Lew AE, Jorgensen WK. Molecular approaches to detect and study the organisms causing bovine tick borne diseases: babesiosis and anaplasmosis. *Afric J Biotechnol.* 2005;4 (4), 292-302.
 17. Molad T, Mazuz ML, Fleiderovitz L, Fish L, Savitsky I, Krigel Y, Leibovitz B, Molloy J, Jongejan F, Shkap, V. Molecular and serological detection of *A. centrale*- and *A. marginale*-infected cattle grazing within an endemic area. *Vet Microbiol.* 2006;113, 55–62.
 18. Coetzee JF, Apleya MD, Kocan KM, Rurangirwa FR, Donkersgoed JV. Comparison of three oxytetracycline regimens for the treatment of persistent *Anaplasma marginale* infections in beef cattle. *Vet Parasitol.* 2005;127, 61–73.
 19. Torioni de Echaide S, Knowles DP, McGuire TC, Palmer GH, Suarez CE, McElwain TF. 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 5. *J Clin Microbiol.* 1998;36(3),777-82.