Differentiation of sheep *Theileria spp*. and *Babesia spp*. by Polymerase Chain Reaction

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Abstract: The diagnosis of piroplasms is achieved by staining methods such as Giemsa, feulgen or Methyl-green-puronin on blood smear or tick salivary gland. These methods are not specific and could be accompanied with some complications, especially in the carrier animals. In contrast to these methods, polymerase chain reaction is more sensitive and specific. Different sources of blood samples, such as EDTA-blood, ethanol blood fixed, Giemsa stained blood smear or from native were used for DNA extraction. Phenol/chloroform extraction method, TriPure method and rapid DNA-Isolation method (Kit) were applied to compare DNA-isolation. Protozoan DNA could be amplified using extracted DNA from EDTA- blood with all three methods. DNA extracted from blood fixed in ethanol could be successfully analyzed using Phenol/chloroform extraction method and rapid DNA extraction method (kit). But in the case of extracted DNA from native or stained blood smear, protozoan DNA could be demonstrated only when the DNA was extracted using rapid DNA-extraction method (kit). The results showed that the amount of blood for the PCR analysis revealed that less than 10 µl blood could be sufficient to detect protozoan parasites in the infected animals. These results suggest that it is possible to analyze and control the already stained and registered blood smear from infected animals. Furthermore, the results facilitated possibility to develop simpler method for the collection of samples than the conventional methods. *J.Vet.Res. 62,2:15-20,2007.*

Key words : *Theileria*, *Babesia*, diagnosis, DNA, PCR.

Introduction

Theileria and *Babesia* are tick-borne protozoan parasites, which cause a high economic loss in domestic ruminants in Iran (Anwar 1974). Interestingly, the purpose of review of Tick-borne diseases has increasingly been recognized throughout the world as public health problems (Zintl *et al.,* 2003, Persing *et al.,* 1995). For example, *Babesia* divergens, the main agent of bovine babesiosis in Europe, is not only a cause of significant loss to the cattle industry, it can also infect immunocompromised humans, causing medical emergencies characterized by rapid fulmination and parasitemia that may exceed 70% (Zintl *et al.,* 2003). Infection of human with a *babesia*-like organism with the phylogenic character of theileria species was already described (Persing *et al.,* 1995).

At present, an exact differentiation between these parasites in small amount of material is not possible, due to the limited capacity of diagnostic and differentiation techniques, which are currently based mainly on morphological differences between these various pathogens. The most used method is the Giemsa-staining of blood smear, which could be accompanied with some technical problems and in some cases, needs special diagnostic knowledge. In certain cases, serological methods such as the indirect fluorescence antibody test have also been applied (Jianxun and hung 1997; Leemans *et al.,* 1997).

One of the discussed problems in protozoan infections is to determine and characterize the

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transmitter agent. Since many experiments performed with the salivary gland smear using unspecific Methyl-green-puronin or feulgen-staining method, the discussion about the transfer vector in some cases remains unanswered. For example: Uilenberg (1997) described that Theileria lestoquardi transmit by Hyaloma anatolicum anatolicum, while some other investigators believe that it may be transmitted by Repicephalus bursa or most probably also by Repicephalus sanguinius (Dschunkowsky and Urodschevich, 1924, Ramzi *et al.,* 2003). With respect to the complicated preparation of for the analysis suitable samples, transport of sample to the specialized laboratories and their subsequent specific analysis, some easy molecular-biological methods were taken into consideration and we showed for the first time a method for the differentiation of theileiosis or babesiosis using PCR with the material from already stained and registered blood smear from infected animals.

Materials and Methods

Thirty peripheral blood samples from sheep with suspicion of theileriosis or babesiosis were analyzed. Ten of them were prepared with EDTA and 10 samples were fixed with ethanol (1ml blood/3 ml absolute ethanol) and 10 native or Giemsa stained blood smears. All tissues had been obtained with consent given according to the institutional guidelines.

DNA Extraction Using Phenol/Chloroform: In the case of more than 100 µl blood, erythrocytes were first lysed in 0.155 M NH_4Cl, 0.01 M $KHCO_3$ and o.1 mM EDTA for 10 minutes, washed twice with PBS at 1000 x g and the pellet was resolved in 200 µl of 10 mM NaCl, 20 mM Tris-HCl pH 8.0 and 1 mM EDTA. After that 20µl proteinase K (10 mg/ml) was added and the sample was incubated for 10 minutes by 55°C to digest the proteins. After addition of equal volume of Tris-HCl pH 8.0 saturated phenol, the sample was gently vortext and centrifuged at 12000 rpm for 15 minutes. Upper liquid phase was transferred to the clean tube. The last step was repeated once with phenol/ chloroforme/ Isoamylalcohol (25/24/1) to eliminate proteins and once with chloroforme/Isoamylalcohol (24/1) to remove rest phenol in the solution. Finally, 1/10 volume of the sample from 6M Na-acetat and 2.5 volume of the sample from ethanol 96% were added and the sample was incubated for 20 minutes by -70°C or over night by -20°C. DNA was then precipitated by 12000 rpm and after washing of pellet with 70% ethanol, the pellet was dried and dissolved in TE-buffer (10 mM Tris-HCl, 0.1 mM EDTA pH 8.0).

DNA Extraction Using TriPure Isolation Reagent: DNA extraction was performed according to the manufacturer·s instruction (Roche, Germany). Briefly, 5 - 10 x 10^6 peripheral nucleated blood cells were homogenized with 1 ml TriPure Isolation reagent. After addition of 0.5 ml Isopropanol, the cell lysate was first incubated for 10 minutes at room temperature and than centrifuged for 10 minutes by 12000 x g at 2-8°C. Three hundred µl absolute (95-100%) ethanol were added to the lower and intermediate phase. DNA was precipitated after incubation of 2 - 3 minutes at room temperature by 2000 x g at 2-8°C centrifugation. The pellet was then washed twice with 0.1 M sodium citrate in 10% ethanol and subsequently it was dissolved in TE-buffer.

DNA Extraction Using MBST- Kit: In contrast to the last two methods, this method was based on the specific binding of DNA to the carrier. Therefore, in this system, neither phenol/chloroform nor DNA precipitation was used. In the case of more than 100 µl blood, erythrocytes were first lysed in blood samples using Erys-Lysing-Buffer. DNA was extracted using a DNA isolation kit (MBST, Germany/Iran) according to the manufacturer·s instructions. Briefly, the cells were first lysed in 100 µl lysis buffer and the proteins were degraded with 10 µl proteinase K for 10 minutes at 55°C. After addition of 270 µl Bindings buffer and incubation for 10 minutes at 70°C, 320 µl ethanol (100%) was added to the solution and after vortexing, the complete volume was transferred to the MBST-column. MBST-

column was first centrifuged and was then washed twice with 500 µl washing-buffer. Finally, DNA was eluted from the carrier with Elution buffer.

DNA Extraction from Ethanol Fixed Blood: Ethanol fixed blood was first centrifuged for 20 minutes by 13000 rpm at 8°C and then air dried by converting of tube. The pellet material was dissolved in lysis buffer (obtained from Phenol/chloroform method or from MBST kit) using proteinase K for various time interval, until the solution was homogenized. Finally, DNA was extracted according to the protocol of phenol/chloroform method or to the MBST manufacturer·s instruction.

DNA Extraction from Blood Smears: Blood smears were separately cleaned shortly first in acetone and then in ethanol. To avid contamination, each blood smear was treated in the separate vessel. Half of blood smear before and after Giemsa staining was dissolved in 100 µl saturated phenol or 100 µl TriPure reagent or 100 µl Lysis buffer of MBST kit. DNA extraction was performed as described above and was dissolved in 100 µl TE buffer.

Polymerase Chain Reaction: Approximately 100 to 500 ng DNA or 5 to 10 µl DNA solution in the case of extraction from blood smear was used for the PCR analysis. PCR was performed in 100 µl total volume including 1 x PCR buffer, 2.5 U Taq Polymerase (Cina gene, Iran), 2 µl of each primer (20 mM, MWG, Germany), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermentas) and 1.5 mM $MgCl_2$ in automated Thermocycler (Eppendorf, Germany) using the following program: five minutes incubation at 95°C to denature double strand DNA, 35 - 38 cycles of 45 second at 54 - 58°C (annealing step), 45 second at 72°C (extension step) and 45 second at 94°C (denaturing step). Finally, PCR was completed with the additional extention step for 10 minutes. Water was used as negative control in each PCR reaction. The DNA from theileria schizont infected cell line and in vivo experimental infected erytrocytic form of *babesia* were used as positive controls for corresponding primers.

PCR products were analyzed on 1.8% Agarose gel in 0.5 x TBE buffer and visualized using Ethidium bromide and UV-illuminator. Primers are listed in Table 1.

Results

DNA was extracted from different prepared peripheral blood of infected sheep with theileria or *babesia* using phenol/chloroform method, TriPure reagent and MBST-Kit.

DNA could be isolated from leukocytes of 1 ml or more peripheral blood with all three methods. Visualization on agarose gel showed that the amount of DNA extracted with MBST-Kit was much more than with phenol/chloroform method or TriPure reagent. In the case of isolation of DNA from 100 µl or less than 100 µl peripheral blood, only DNA was detectable on agarose gel by MBST-kit.

DNA could be only extracted from ethanol fixed peripheral blood, when fixed material was first completely air dried and was treated with proteinase K. Since TriPure reagent did not include proteinase K, therefore DNA extraction with this method had to be modified.

The amount of isolated DNA from ethanol fixed peripheral blood was comparable to the amount of isolated DNA from above mentioned peripheral blood.

Extraction for further analysis suitable DNA from blood smear was only feasible with MBST-Kit. In this coherence, we could not find any negative effect of Giemsa staining on DNA extraction. The quality of DNA extracted from blood smear before and after Giemsa staining was comparable.

PCR analysis showed that generally specific PCR-products for Theileria and *Babesia* could be amplified with the DNA extracted with all three methods (Fig. 1).

Only in some cases, purification of DNA needed to be repeated, when it was extracted with Phenol/chloroform method or with TriPure reagent. It is known that the trace of phenol or high salt concentration inhibits enzyme activity.

PCR analysis of extracted DNA from infected blood smear revealed specific PCR product with

Table1. Primer used for the amplification of the DNA from *Theileria spp*. and *Babesia spp*. are species specific (Shayan *et al.,* 1998, Schnittger *et al.,* 2000 and Dalrymple *et al.,* 1993). The sequences of primers were designed from the genes registered in National Centre for Biotechnology Information (NCBI). Locus, Definition and Accession of the genes are given in the table.

no.	name	gene	nucleotide sequences	PCR-Product
1	SA^{288}-sense (Shayan *et al.,* 1998, Schnittger *et al.,* 2000)	Heat shock protein 70 AY271268 NCBI	5· TGTCAAGGAGGCCTCAAATTA 3·	275 bp
2	SA^{288}-antisense		5· TTTGACTTTGAATAGGCTGCC 3·	
3	Theileria Lestoquardi-sense	Theileria lestoquardi ms1-2 gene AJ006448 NCBI	5·GTTACTCTCACTTCATGTGAG 3·	669 bp
4	Theileria lestoquardi-antisense		5·GGAGAACTTGTCGACAGCTGG 3·	
5	*Babesia*-sense (Dalrymple *et al.,* 1993)	Rhoptry protein gene M91176 NCBI	5· CAGGATTGCTTTCGCAACAAAG 3·	239 bp
6	*Babesia*-antisense		5· CCTTGACATAACCGGCGAGG 3·	

primer derived from genes specific for Theileria or *Babesia*. Interestingly, 5 to 10 µl of DNA solution (total 100 µl) was sufficient for the analysis (Fig. 2).

Discussion

Control and management of livestock health could be understood as both the site of gold coin for successful and healthy economy in stock-farming. In this coherence, control of tick-borne diseases plays a prominent role. One of the most important diseases in small ruminants is the infection with protozoan parasites Theileria and *Babesia*, which cause annually high economic losses in worldwide. Furthermore, the purpose of review of the Tick-borne diseases has been increasingly recognized throughout the world as public health problems. Recently, babesiosis also has been recognized frequently in human, thereby highlighting the importance of tick-borne-diseases generally.

Characterization of transmitting vectors, early diagnosis, therapy and prevention such as vaccination belongs to the main components of control and management of livestock health.

The suitable tools are needed to realize these presuppositions. Early diagnosis requires a simple, but precise method with the capability to recognize infecting agents in very low density in ticks and/or in animals.

Taking of high number blood samples, cold storage and sending of probes from farm to the laboratory are time and cost intensive and are accompanied with some problems. To minimize these problems and unsuitability for veterinarians and livestock, we developed for the first time a new diagnostic system. For this aim, we have first isolated DNA from EDTA-peripheral blood, peripheral blood fixed in ethanol and from blood smear of Theileria or *Babesia* infected sheep, in which the infection has been already determined using Giemsa staining. For the isolation of DNA, we used three DNA extraction methods (Phenol/Chloroform method, TriPure reagent, MBST-Kit).

Our results showed, indeed DNA could be extracted from peripheral blood with all three methods, when it was approximately 1 ml or more. But the quantity of extracted DNA with MBST method was always higher than the other two methods. This is more probably due to the specific binding of DNA to the carrier placed in the MBST-column. This assumption could be supported by extraction of DNA from less than 100 µl blood, since in comparison to the MBST-Kit, not sufficient DNA could be extracted with Phenol/ chloroform method or with TriPure reagent as we analyzed by agarose gel electrophoresis and staining with ethidiume bromide. Furthermore, according to the manufacturer·s instruction (Roche, Germany) the use of glycogen (5 -10 µg) is recommended as a carrier to assist RNA precipitation for extraction of less than one million nucleated cells. That means that extraction of DNA/RNA on the basis of phenol is not proportional to the amount of the pure DNA.

The results of DNA extraction from ethanol fixed peripheral blood were comparable to the above mentioned results, but solublization and proteinase K

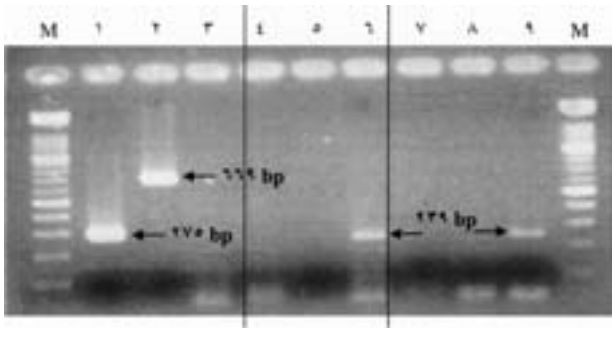

Figure 1: DNA was extracted from infected blood samples and analyzed with Primers specific for *Theileria spp*. (SA[288] lanes 1, 4, 7 ; Theileria lestoquardi ms1-2 gene lanes 2, 5, 8) and *Babesia spp*. (Rhoptry protein gene lanes 3, 6, 9). M is 100 bp marker.
Analyzed samples from sheep with theileriosis (lanes 1, 2 and 3), samples from 2 sheep with babesiosis (sheep 1: lanes 4, 5 and 6; sheep 2: lanes 7, 8 and 9)

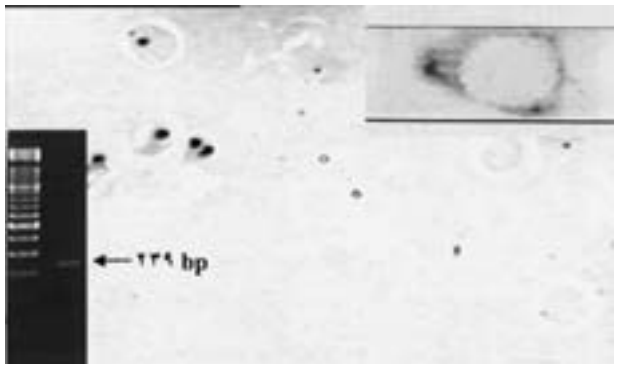

Figure 2: Micrograph of giemsa-stained blood smear shows *babesia* particles.
Upper right: the spot of the same smear after DNA extraction using MBST-Kit.
Lower left: the gel analysis of the extracted DNA amplified using primer specific for *Babesia spp*.

treatment of samples were much more time intensive. Only in the case of TriPure reagent, the procedure must be modified and cell lysis step and proteinase K step must be added. Our results showed that ethanol fixation could be used as a suitable preparation method, when the subsequent analysis may be achieved on the DNA basis. The advantage of this method to the untreated samples is mainly in the long time conservation of materials.

One of the most prominent results was to determine the infection with already stained blood smear using PCR technique. Determination and characterization of piroplasms are usually performed on blood smear of corresponding animals using Giemsa staining. Therefore, the preparation of blood smear and its subsequent staining are considered as the most important steps. In some cases, the analysis on blood smears is not exactly possible either due to the thickness, dirtiness or not well staining of blood smears. Our study has been focused on exactly these problems and we showed that the extraction of suitable DNA from already stained blood smears for the molecular analysis is possible. Since each blood smear is as a rule prepared with approximately 10 µl blood, DNA extraction for analysis required absolute presuppositions, which were only fulfilled by MBST-kit. Figure 2 showed a typical character for such analysis, in which babesiosis was not with conventional method to diagnose, but with PCR was absolutely to determine. The tick salivary gland smear, which has been already stained with feulgen (Razmi *et al.,* 2003), should be examined on the molecular level using PCR technology, to confirm the feulgen positive bodies as piroplasmic protozoan.

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References

1. Anwar, M. (1974) Geographical distribution of blood protozoan parasites of ruminants in Iran. Bult. Off. Int. Epiz.81: 793-798.
2. Zintle, A., Mulcahy, G., Skerrett, H.E., Taylor, S.M. and Gray, J.S.(2003) *Babesia* divergens, a Bovine Blood Parasite of Veterinary and Zoonotic Importance. Clin. Microbiol. Rev.16:622-636.
3. Dalrymple, B.P., Casu, R.E., Peters, J.M., Dimmock, C.M., Gale, K.R., Boese, R. and Wright, I.G. (1993) Characterisation of a family of multi-copy genes encoding rhoptry protein homologues in *Babesia*

bovis, *Babesia* ovis and *Babesia* canis. Mol. Biochem. Parasitol. 57: 181-192.

4. Dschunkowsky, E., Urodschevich, V. (1924) Theileriosis in goats, sheep and cattle with description of Theileria hirci from Serbia. Parasitol. 16: 107 - 110.
5. Jianxus, L., Hong, Y.(1997) Theileriosis of sheep and goats in china. Trop. Anim. Health Peod, 29 (suppl.), 8S-10S.
6. Leemans, I., Hooshmand-Rad, P., Uggla, A. (1997) The indirect fluorescent antibody test based on schizont antigen for study of the sheep parasite Theileria lestoquardi. Vet. Parasitol. 69: 9-18.
7. Persing, D.H., Herwaldt, B.L., Glaser, C., Lane, R.S., Thomford, J.W., Mathiesen, D., Krause, P.J., Phillip, D.F. and Conrad, PA. (1995) Infection with a *babesia*-like organism in northern California. N. Egl. J. Med. 332: 298-303.
8. Ramzi, G.R., Hosseini, M., Aslani, M.R. (2003) Identification of tick vectors of ovine theileriosis in an endemic region of Iran. Vet. Parasitol. 116: 1-6.
9. Shayan, P., Biermann, R., Schein, E., Gerdes, J. and Ahmed, J.S.(1998) Detection and differentiation of Theileria annulata and Theileria parva using macroschizont-derived DNA probes. An. New York Acad. Sci.29: 88-95.
10. Schnittger, L., Shayan, P., Biermann, R., Mehlhorn, H., Gerdes, J. and Ahmed, J.S. (2000) Molecular genetic characterization and sub cellular localization of Theileria annulata mitochondrial heat-shock protein 70. Parasitol. Res. 86: 444-452.
11. Uilenberg, G. (1997) General review of tick-borne diseases of sheep and goats worldwide. Parasitol. 39: 161 - 165.