RAPD-PCR Analysis of *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum* (Acari:Ixodidae) of Iran

Abdigoudarzi*[1,2], M., Kamali, K.,[1] Dinparast Djadid, N.[3] and Saboori, A.[4]

1. Agriculture Faculty, Tarbiat Modarres University, Tehran, Iran

2. Razi Vaccine & Serum Research Institute, P.O.Box 11365-1558, Tehran, Iran

3. Pasteur Institute of Iran, Tehran, Iran

4. Agriculture Faculty, Tehran University, Tehran, Iran

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Summary

Eighteen RAPD primers, from AB1 and OPA1 series, M13 and a Pz primer, were used to amplify polymorphic DNA of *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum* for the first time. Rapid, Zahler and Collins methods and a specific kit were used for DNA extraction. 16 primers out of 18 and the Pz primer could successfully amplify the genome of *H.anatolicum anatolicum* and *H.marginatum*. Pattern of the bands were reproducible and species specific for these two ticks. The RAPD-PCR for its simplicity, rapidity, and ability to reveal variation at the DNA level is an additional taxonomic tool for the study of ticks.

Key words: *Hyalomma anatolicum anatolicum*, *Hyalomma marginatum*, RAPD-PCR, taxonomy

Introduction

Ticks (Acari:Ixodidae) are blood feeding obligatory external parasites of human and animals. They have got their medical and veterinary importance due to their direct blood feeding and their ability for transmitting viral, bacterial, and protozoan

*Author for correspondence. E-mail:sepehr2001ir@yahoo.com

pathogens to their hosts. Regarding the problems in their taxonomy and viewing morphological characters it has been decided to use polymerase chain reaction (PCR) techniques for their identification. Now, there are several ways using specific and random molecular markers searching for species specificity or different strains in specific population of species. The random amplified polymorphic DNA (RAPD) PCR technique was first described by Williams *et al* (1990) to amplify arbitrary regions of a genome using single primer. Lan *et al* (1996) used the technique for detecting DNA polymorphism in *Boophilus microplus* (Ixodidae) in China. There is another interesting RAPD analysis report on ticks that the researchers found variations within the RAPD profiles between susceptible and acaricide resistant *Boophilus microplus* ticks using 120 primers (Hernandez *et al* 1998).

There are some reports of PCR inhibition due to the presence of residual haem pigments or other impurities in the hemolymph of ticks (Panaccio & Lew 1991). DNA derived from non-target organisms present on the surface of ticks may give amplicons with some primers (Sparagano *et al* 1999, Barthold 1998). Several techniques have been used for DNA extraction from ticks and the pathogens they carry including crushing tick eggs, larvae, or nymphs by disposable needles (Zahler *et al* 1995), freezing and crushing individual ticks in a homogenized buffer (Black & Piesman 1994), snap freezing ticks in liquid nitrogen (Crampton *et al* 1996). Hubbard *et al* (1995) developed an extraction method employing a diatomaceous earth suspension for alcohol-preserved ticks, which eliminates blood constituents inhibiting PCR. A summary of DNA extraction methods and references is given by Sparagano *et al* (1999).

In this study regarding permanent problems for tick diagnosis at the species and subspecies levels specially for identification of *H.excavatum complex* members which are prevalent ticks in Iran, two famous related species (i.e. *H.anatolicum*

anatolicum and *H.marginatum*) were collected from field and RAPD-PCR was done using eighteen RAPD primers for the first time.

Materials and Methods

Tick rearing method. Fully engorged female ticks were collected from cattle during September in Boushehr province located in Southern parts of Iran. The ticks were identified by traditional morphological methods and their life cycle parameters were studied under laboratory conditions, then they were kept individually in vials at 28±1°C and 75-80% relative humidity (r.h.). Eggs from individual ticks were collected four days after ovipositon and stored at -70°C before experiments. Some of the eggs were kept at 28°C and 75% r.h. until hatching of larvae. Larvae were put in ear bag on rabbit for blood feeding. Engorged nymphs were transferred to incubator, molting has occurred and viable male and female ticks were used for DNA extraction individually.

DNA extraction. Regarding to the related background of molecular experiments on ticks, three DNA extraction methods including Rapid method (Gloor *et al* 1993) Zahler method (Zahler *et al* 1997) and Collins method (Collins *et al* 1989), and a DNA extraction kit (Biotools, Spain) were selected and used for DNA extraction from viable ticks. The DNA concentrations were 335ng/µl and 200ng/µl for *H.anatolicum anatolicum* and *H.marginatum* respectively.

RAPD-PCR analysis. Eighteen primers from AB1 and OPA1 series, M13 and Pz primer (CGGCCCGGTA) (Lan *et al* 1996) were selected. They were M13 (5'-GTAAAACGACGGCCAGT-3'), OPA1 (5'-CAGCCCCTTC-3'), AB1-01 (5'-GTTTCGCTCC-3'), AB1-03 (5'-CATCCCCCTG-3'), AB1-04 (5'-GGACTGGAGT-3'), AB1-06 (5'-TGCTCTGCCC-3'), AB1-7 (5'-GGTGACGCAG-3'), AB1-9 (5'-

TGGGGGACTC-3'), AB1-11 (5'-GTAGACCCGT-3'), AB1-12 (5'-CCTTGACGCA-3'), AB1-13 (5'-TTCCCCCGCT-3'), AB1-15 (5'-GGAGGGTGTT-3'), AB1-16 (5'-TTTGCCCGGA-3'), AB1-17 (5'-AGGGAACGAG-3'), AB1-18 (5'-CCACAGCAGT-3'), AB1-19 (5'-ACCCCCGAAG-3'), AB1-20 (5'-GGACCCTTACGGACCCTTAC-3').

PCR assay was performed in 50µl of a reaction mixture contained template DNA, primers at 0.5µM each, dNTPs at 200µM each, 2mM $MgCl_2$, 1x PCR buffer, and 5U of *Taq* DNA polymerase (Cinnagen company, Iran). The mixture was covered by a layer of mineral oil. PCR amplification conditions were 7min of denaturation at 92.0°C, followed by 45 cycles of 92.0°C for 30s, 36°C for 30s, 70°C for 90s and a final extension of 70°C for 5min. A sample of 20µl of amplified PCR products was electrophoresed in 1.5% agarose gel with 0.5µg ethidium bromide/ml. Gels were photographed under UV illumination with gel documentation system.

Results

DNA extraction. DNAs of adult stages of *H.anatolicum anatolicum* and adult stages of *H.marginatum* (Qome strain) were extracted using Rapid, Collins and Zahler methods respectively. Using Collins method we could not extracted DNA from ticks. Accordingly, DNA from adult *H.marginatum*, two batches of eggs laid by *H.anatolicum anatolicum* (kept frozen for one year at -70°C) and *H.marginatum* (fresh laid eggs), and partially engorged nymphs of *Boophilus annulatus* was successfully extracted by a DNA purification kit (Biotools, Spain).

RAPD-PCR of H.anatolicum anatolicum and H.marginatum eggs. The RAPD patterns for *H.anatolicum anatolicum* and *H.marginatum* eggs obtained with Pz primer are shown in figure 1. The photos prepared by a gel documentation system after 2h. The pattern of bands for the two species was different and species specific.

The patterns were reproducible.

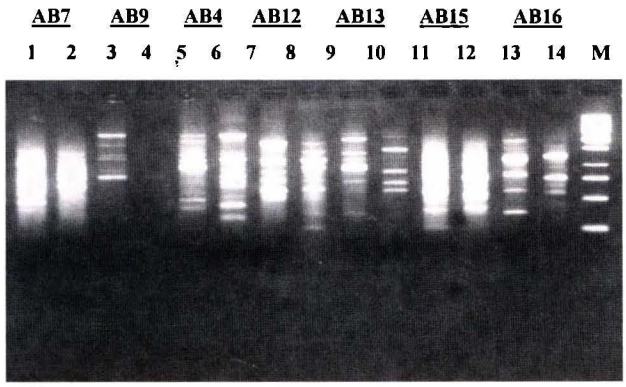

Figure 1. *Agarose gel electrophoresis of RAPD-PCR products on extracted DNA from eggs laid by H.anatolicum anatolicum (left lanes) and H.marginatum (right Lanes) and primer Pz*

RAPD-PCR of H.anatolicum anatolicum and H.marginatum adult stages. The RAPD fingerprint patterns of the two species obtained with primers AB7, AB9, AB11, AB12, AB13, AB15, and AB16 were analyzed. The pattern obtained with each primer was species specific (Figure 2). Primer screening was done for all selected primers and 16 out of 18 primers could amplify the polymorphic DNA of ticks. The extracted DNA from adult ticks was successfully used for amplification of a specific ribosomal marker for ticks (data not shown).

Discussion

Random amplified polymorphic DNA technique uses a single decamer primer that hybridizes to and amplifies arbitrary regions of a genome. Different species may have different annealing sites and thus give different patterns of bands (Williams *et*

al 1990). After the first report by Williams (1990), RAPD technique has been widely used in many kinds of organisms, including mosquitoes, sandflies, schistosome and malaria parasites, to solve the problems which may be difficult to solve by traditional methods (Lan *et al* 1996). They have shown that RAPD pattern analysis is useful for identification of the organisms. Lan *et al* (1996) reported that 12 and 13 bands could obtain for *H.asiaticum* and *Boophilus microplus* using Pz primer, respectively. They concluded that the primer was suitable for RAPD-PCR of ticks.

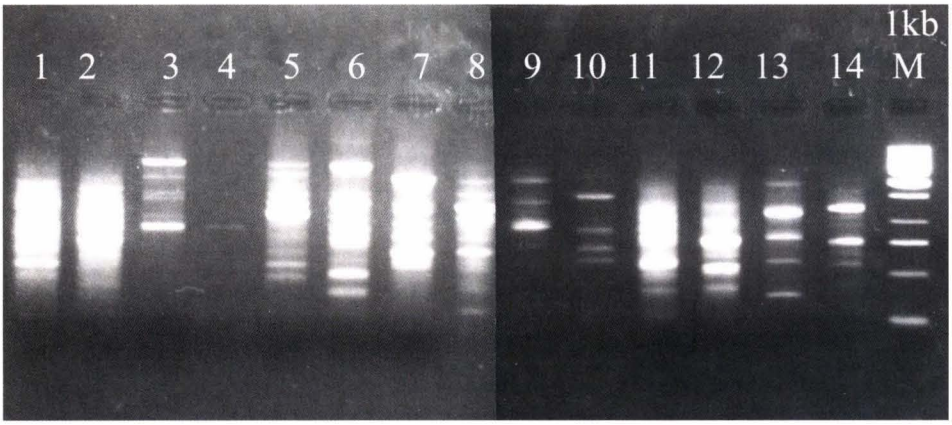

Figure 2. *Agarose gel electrophoresis of RAPD-PCR products on extracted DNA from adult stages of H.anatolicum anatolicum and H.marginatum using seven different RAPD primers*

In this study described here, we could successfully use the Pz primer for the two Iranian species, *H.anatolicum anatolicum* and *H.marginatum*. Furthermore, 17 RAPD primers were also used for rapid detection of the two species and two separate RAPD fingerprint patterns were recognized by all of them. Based on the reproducible patterns obtained for *Hyalomma* ticks, the primers were specific for the species. Although, more detailed studies are needed, the primers should be useful candidates

for identification of the ticks. This study reveals the potential of RAPD-PCR for discriminating ticks at the species level and to our knowledge; this is the first report of primers that can be used for the determination of *H.anatolicum anatolicum* and *H.marginatum*. Additional study for searching intraspecific variations in specific geographical populations of Iranian Ixodidae ticks is going to be done in future.

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