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Original article

PCR-RFLP DETECTION OF *HAEMOPROTEUS* SPP. (HAEMOSPORIDA: HAEMOPROTEIDAE) IN PIGEON BLOOD SAMPLES FROM IRAN

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Summary

Tavassoli, M., B. Esmaeilnejad, F. Malekifard & K. Mardani, 2018. PCR-RFLP detection of *Haemoproteus* spp. (Haemosporida: Haemoproteidae) in pigeon blood samples from Iran *Bulg. J. Vet. Med,* **21**, No 4, 429–435.

This study was carried out to determine *Haemoproteus* spp. infection in pigeons in Iran. Blood samples collected from pigeons were examined for *Haemoproteus* spp. using stained blood smears and polymerase chain reaction (PCR). For PCR, DNA was extracted from blood samples and a fragment of 617 bp in size subjected to PCR using HAEMF and HAEMR2 derived from cytochrome *b* gene of the parasite mitochondrial genome. A total number of 93 blood samples from pigeons were examined for *Haemoproteus* spp. of which 13 (13.97%) samples were positive in stained blood smears for *Haemoproteus* spp. and 27 (24.73%) were positive in PCR. Digestion of PCR product with *Alu*I restriction endonuclease generated only one distinct pattern for all positive samples, which is indicative of identical *Haemoproteus* spp. presence in infected pigeons. The results also revealed that PCR had higher sensitivity in detecting *Haemoproteus* spp. in pigeons.

Key words: *Haemoproteus* spp., Iran, PCR-RFLP, pigeon

INTRODUCTION

Haemoproteus spp. is the most prevalent and widespread avian haemoparasite in the world (Iezhova *et al.,* 2010). The natural hosts of *Haemoproteus* spp. include many species of wild pigeons, domestic pigeons *(Columba Iivia domestica),* turtle doves, mourning doves *(Zenaidura macroura),* and other wild bird species (Soulsby, 1984). *Haemoproteus* are transmitted by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae) (Valkiūnas, 2005). Severe *Haemoproteus* infection can result in depression, haemolytic anaemia and anorexia in stressed or immunocompromised birds (Campbell, 1994). The prevalence of this vector-borne disease is complicated by host-vector-parasite interactions (Knowles *et al.,* 2011). Historically, avian malaria has been detected by microscopic examination of blood smears (Fallon *et al.,* 2003).

Diagnosis of *Haemoproteus* spp. can be achieved by microscopic examination of Giemsa-stained blood smears and clinical signs in acute phase of the disease. On the blood smears, *Haemoproteus* appear as dumbbell-shaped structures in erythrocytes (Iezhova *et al.,* 2010). Moreover, examination of Giemsa-stained blood smear can provide estimate of parasite intensity (Waldenström *et al.,* 2004). However, this procedure has several drawbacks, in particular that it is time-consuming and needs well-trained personnel to obtain reliable data (Richard *et al.,* 2002). After acute infections, recovered birds frequently sustain subclinical infection, and in such cases, accuracy of microscopical diagnostic methods is not satisfactory (Waldenström *et al.,* 2004; Valkiūnas *et al.,* 2005; Bentz *et al.,* 2006). According to Valkiūnas *et al.* (2008), PCR tests were 3- to 4-fold more sensitive than microscopy for detecting chronic blood parasite infection. In addition, samples examined by using both PCR-based technique and blood smear showed approximately 10-fold difference in prevalence of hematozoa (Durrant *et al.,* 2006).

The aim of the present study was to determine the infection rate of *Haemoproteus* spp. in pigeons from different regions of Iran by microscopic examination of stained blood smears and PCR method. PCR-restriction fragment length polymorphism (PCR-RFLP) was also applied for identification of *Haemoproteus* spp. lineages.

MATERIALS AND METHODS

Blood samples and blood smear staining

Blood samples were collected from 93 adult free living pigeons from five provinces of Iran (West and East Azerbaijan, Kurdistan, Fars and Kermanshah). The age range of pigeons was 6 months to 3 years. A blood sample from each bird was transferred into vacutainers tubes containing EDTA and stored in a freezer after collection. Blood smears were immediately prepared. Smears were air dried, fixed in methyl alcohol and stained in a 10% working solution of Giemsa stain. Each blood smear was examined under ×1000 microscopic magnification and at least 100 to 200 microscopic fields were investigated.

DNA extraction from blood samples

DNA was extracted from whole blood and Giemsa-stained samples. DNA extraction was performed as previously described (Barker *et al*., 1992). Briefly, 500 µL of PCR lysis mixture (0.22% NaCl, 0.015% saponin, 1 mM EDTA) was added to 50 µL of blood, and the mixture was then centrifuged at 10,000×g for 1 min. Pellets were washed three times with 0.5 mL of PCR lysis mixture, resuspended in 100 µL of PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8], 0.5% Tween 20, 100 mg of proteinase K per mL), and incubated at 56 ^{o}C for 1 h. After 10 min incubation at 95 ^{o}C, 5 µL was taken for the PCR assay.

PCR reaction and RFLP analysis

For PCR amplification of the cytochrome *b* region of the mtDNA spanning the 617 bp amplicon, forward and reverse primers HAEMF (5′-ATGGTGCTTTCGATATA TGCATG-3′) and HAEMR2 (5′-GCA TTATCTGGATGTGATAATGGT-3′) as described by Bensch *et al*. (2000) were used. The PCR reaction was carried out in 25 µL reaction volume, containing 50 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM each of primer, 2.5 µL of 10× PCR Buffer (Fermentas, Cinnagen, Iran), 2 mM of magnesium chloride, 1.0 U Taq DNA polymerase (Fermentas, Cinna-

Table 1. Prevalence of infection among pigeons in five provinces of Iran – number (percentage) and confidence intervals

Province	Methods			
	Microscopy		PCR	
	Positive/ Examined	Prevalence % (95% CI)	Positive/ Examined	Prevalence % (95% CI))
West Azarbaijan	6/35	17.14 (8.1–32.68)	11/35	31.42 (18.55–47.98)
East Azarbaijan	2/18	11.11 (3.1–32.8)	5/18	27.77 (12.5–50.87)
Kurdistan	2/15	13.13 (3.73–37.88)	4/15	26.66 (10.9–51.95)
Fars	1/12	8.33 (1.5–35.38)	3/12	25.00 (8.1–32.68)
Kermanshah	2/13	15.38 (4.32–42.23)	4/13	30.76 (12.68–57.63)
Total	13/93	13.97 (8.36–22.46)	27/93	29.03 (20.78–38.94)

gen, Iran) and 3 μL of extracted DNA as template. The PCR profiles involved an initial denaturation for 3 min at 94 °C followed by 35 cycles of incubation at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s with a final extension at 72 °C for 10 min (Waldenstrom *et al*., 2004). The obtained PCR products were separated on 2% agarose gel and were observed using ultraviolet transillumination. Positive controls consisted of DNA from birds known to be infected through microscopic examination of blood smears. For negative controls 5 μL of distilled water was used. After PCR amplification, the PCR products were digested for 16 h by 10 U of *Alu*I restriction enzymes and then analysed by 2.0% agarose gel in 0.5× TBE buffer for 1 h, followed by ethidium bromide staining.

Statistical analysis

Data were analysed using SPSS (Version 17; SPSS Inc., Chicago, USA). A value of $P<0.05$ was considered as statistically significant.

RESULTS

Microscopic investigation

Microscopic examination of blood smears revealed that 13 (13.97%) of the blood samples collected from different regions were positive for *Haemoproteus* spp., of which 6 (17.14%), 2 (11.11%), 2 (13.13%), 1 (8.33%) and 2 (15.38%), positive samples belonged to West Azarbaijan, East Azarbaijan, Kurdistan, Fars and Kermanshah provinces, respectively (Table 1).

PCR-RFLP assay

All blood samples were subjected to PCR assay using extracted DNA from blood samples as template for amplifying a fragment of 617 bp in size using specific primers. A number of 27 (24.73%) blood samples were considered positive by PCR (Table 1, Fig. 1).

The 617 bp PCR amplicons were digested using restriction endonuclease *Alu*I aiming to detect variation in nucleotide sequences of the cytochrome *b* region of mtDNA. The results indicated that *Alu*I

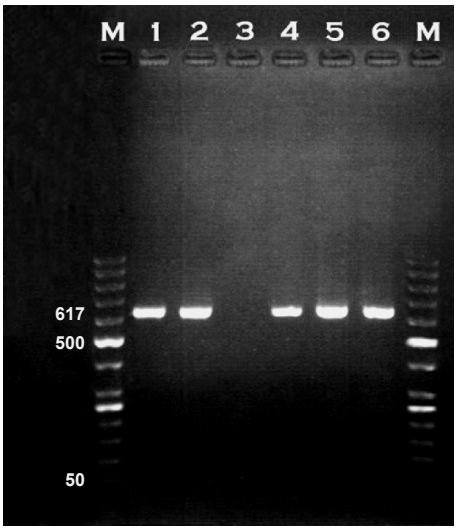

Fig. 1. Agarose gel electrophoresis of amplified PCR products from cytochrome *b* gene of pigeons infected with *Haemoproteus* spp. using primer set HAEMF/HAEMR2. Lanes: 1, 2, 5 & 6: positive samples, lane 3: negative control, lane 4: positive control, lane M: 50 bp molecular size markers (Fermentas, Germany).

digested all PCR products into 3 fragments of 49 bp, 241 bp and 327 bp (Fig. 2) generating only one unique pattern for all isolates. The RFLP analysis revealed that there was no variation in the PCR products obtained from all isolates based on *Alu*I enzyme.

DISCUSSION

Microscopic examination of Giemsa stained blood smears is the common method for diagnosis of *Haemoproteus* spp. in Iran. There are limited studies on *Haemoproteus* infection based on traditional microscopic examination in Iran. These studies have determined that the most common blood parasite found in pigeons is *Haemoproteus columbae* and the infestation rate varied from 7.3 to 24% (Esmaeili *et al.,* 2006; Fakhar *et al.,* 2010; Youssefi *et al.,* 2010; Dehghani Samani *et al.,* 2013). In this study, both microscopic and molecular examinations have

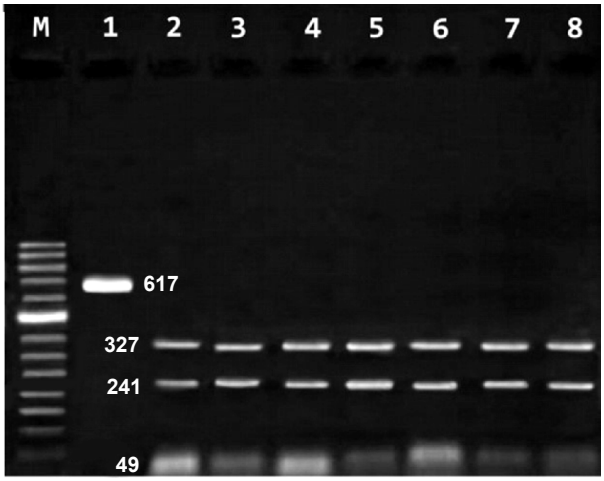

Fig. 2. RFLP analysis of amplified PCR products from *Haemoproteus* spp. cytochrome *b* gene. Lane M: 50 bp molecular weight marker(Fermentas, Germany), lane 1: PCR product of 617 bp from *Haemoproteus* spp., lanes 2–8: RFLP analysis of cytochrome *b* gene PCR products yielded from pigeon blood samples positive for *Haemoproteus* spp.

been performed for the reliable identification and characterisation of the *Haemoproteus* lineage.

Results obtained from this study indicated that 13.97% and 24.73% of the samples were infected with *Haemoproteus* spp. in microscopic examination and PCR-RFLP assay, respectively. The present study is in close agreement with other studies in Iran. However, the infestation rates of *Haemoproteus* in the world are very different. The prevalence of blood parasite in pigeons and birds in Japan (Murata, 2002), Costa Rica (Valkiūnas *et al.,* 2004) and Alaska (Deviche *et al.,* 2001) was lower than 10%. Variations in the prevalence of *Haemoproteus* were probably linked to geographical variations and differences in the feeding habitat and abundance of vehicles of *Haemoproteus* (Nematollahi *et al.,* 2012).

PCR assay had higher sensitivity than microscopic examination for detecting *Haemoproteus* spp. Several studies were carried out for comparative analysis of PCR assay and microscopic examination of blood smears in detecting avian malaria (Fallon *et al.,* 2003; Waldenström *et al.,* 2004; Bentz *et al.,* 2006). The results of this study confirmed those reported by Valkiūnas *et al.* (2008), in that PCR was more sensitive in diagnosing avian malaria than microscopy.

Various types of PCR have previously been used to detect *Haemoproteus* spp. in pigeon blood samples (Bensch *et al.,* 2000; Hellgren *et al*., 2004; Waldenström *et al.,* 2004). In the present study, we used PCR-RFLP method for the detection of avian *Haemoproteus* spp. based on the cytochrome *b* gene of the parasite mitochondrial genome. The PCR-RFLP performed here will be a useful tool for detecting avian *Haemoproteus* spp. and for distinction between different *Haemoproteus* species in birds. This method also allowed detection of mistakes in parasite genus identification more efficiently than smear screening (Bentz *et al.,* 2006). The PCR-RFLP results of this study indicate that one lineage of *Haemoproteus* spp. exists in the provinces under study.

Based on our results, it is concluded that *Haemoproteus* spp. was prevalent among pigeons in Iran. Therefore further studies should be conducted to determine the virulence and distribution of different strains of avian malaria parasites among avian hosts and vectors in other parts of Iran.

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