MOLECULAR EPIDEMIOLOGY OF MALARIA IN ENDEMIC AREAS OF IRAN

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Abstract. In this study, 333 blood samples of malaria cases positive by microscopic test (70.6% male and 29.4% female, $p<0.05$) were investigated. The group included 55 cases (16.52%) from Minab (Hormozgan Province), 116 cases (34.82%) from Iranshahr (Sistan-Baluchesta Province) and 162 cases (48.65%) from Kahnouj (Kerman Province). The results showed 244 cases (73.27%) were diagnosed as *P.vivax*, 87 cases (26.13%) *P.falciparum* and 2 cases (0.6%) showed a mixed infection of both Plasmodia. In a molecular study of the same samples using nested-polymerase chain reaction (nested-PCR), 185 cases (55.6%) were *P.vivax,* 50 cases (15%) *P.falciparum* and 95 cases (29%) both Plasmodia. Comparing the two methods used in this study, the highest rate of infection was found to be *P.vivax.* However, the rate of mixed infections (0.6% microscopy, 29% nested-PCR) varied and depended on the assay used. This indicated that the sensitivity of nested-PCR was greater than microscopic examination, especially for the detection of mixed-infections ($p<0.05$) in the current malaria epidemiology study.

INTRODUCTION

Malaria is one of the most important parasitic diseases in the world. It is estimated that 300 million people are infected every year, with a mortality rate of 2-3 million cases (Hommel, 1996). Malaria is endemic in southeast (SE) Iran with a prevalence of 25.5 per 1,000, with equal proportions of *P.vivax* and *P. falciparum* (Edrissian *et al,* 1993). Many studies on *P. falciparum* cases from SE Iran have been carried out *in vivo* and *in vitro* in malaria endemic areas including Hormozgan, Sistan-Baluchestan and Kerman provinces of Iran (Edrissian *et al,* 1993). The prevalence of malaria in south and southeast (SE) Iran is hypoendemic. There has been a decrease in the incidence rate in recent years due to local drought *eg* from 23,860 cases in 1999 to 19,700 in 2000. The negative consequences for malaria patients is emphasized here, which affect their health, jobs and the economy (Edrissian *et al,* 1993).

Detailed knowledge of the incidence and transmission dynamics of malaria species is central to designing effective malaria control measures (Snounou *et al,* 1993a). Accurate diagnosis in epidemiological studies could be an important measure for therapy and control strategies. Accurate diagnosis is also clearly essential for successful treatment. In both epidemiological data collection and medical diagnosis, microscopy is universally used (Snounou *et al,* 1993a). Its practicality makes it particularly suited for rapid diagnosis. Identification of the species poses few problems to the experienced microscopist, except when one species is numerically dominated by another in a mixed infection. Our aim was to apply a method of parasite detection that can replace routine microscopic examination, without any loss of reliability.

The diagnosis of malaria has traditionally relied on the microscopic examination of Giemsa-stained blood smears. However, even for an expert microscopist, this process is time-consuming and labor-intensive, and it is difficult to identify mixed *P.falciparum* and *P.vivax* infections if only ring stages are present (Kain *et al,* 1993). Microscopy is often the front-line method for the diagnosis of many diseases. It is rapid, simple, inexpensive and well-suited to the mass diagnosis of malaria. It can however, be intensive, it requires experience, and in some cases it may be unable to differentiate between species (Wilson, 1998). Microscopic examination of Giemsa-stained blood smears remains the main malaria diagnosis in endemic areas, but accurate interpretation requires considerable expertise. A rapid and accurate test for Plasmodia infection would facilitate the diagnosis of malaria (Humar *et al,* 1997).

Although several malaria epidemiological studies have been done, the application of molecular methods

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may be useful. This study focussed on using molecular assays for malaria epidemiology in endemic areas of Iran. PCR was used to identify the species of malaria parasites. There is a need for improved molecular diagnostic and epidemiological tools for Plasmodia, particularly in the light of the recent emergence of drug-resistant strains (Tahar *et al,* 1998). PCR has also been used for the rapid detection of malaria parasites in a variety of studies (Tahar *et al,* 1998). Detection of malaria by PCR was evaluated in a field study in Iran. While microscopy is currently the method of choice for detecting malaria, it has several limitations, most notably the time and experience required to process and interpret Giemsa-stained smears (Kain *et al,* 1993).

MATERIALS AND METHODS

This study began in 1998 and was run for two years, between two peaks of malaria infection (spring, autumn) in different geographical areas of Minab (Hormozgan Province), Iranshahr (Sistan-Baluchestan Province) and Kahnouj (Kerman Province) by two microscopic and two molecular assays.

Microscopic method

Thick and thin blood smears were made from finger-prick samples, stained with Giemsa and examined by an expert microscopist. Thin smears were only fixed with ethanol, but both smears stained by 10% Giemsa for 20 minutes and washed in tap water, dried at room temperature (22˚-25˚C), and observed under a light microscope. Up to 200 microscope fields were examined to establish the diagnosis, with which results of the PCR assay were compared (Snounou *et al,* 1993a).

Molecular assay

We described a PCR method for the sensitive detection of the human malaria parasite species. Five ml of peripheral blood were collected from malaria patients and poured into tubes containing ethylene-diamine-tetra-acetic acid (EDTA) in a cold condition (2˚-5˚C). The DNA of all Plasmodia were purified by boiling and exposed to the PCR primers, rPLU5 and rPLU6. The PCR products were reacted with primers, which were specific for prevalent species of human malaria in Iran. Primers used in this study were FAL1 and FAL2 to react specifically with *P. falciparum*, VIV1 and VIV2 with *P. vivax* and MAL1 and MAL2 with *P. malariae* (Snounou *et al,* 1993b). DNA was amplified according to the following thermocycling program: denaturing at 95˚C 1 minute, annealing at 58˚C 2 minutes, and extension at 72˚C 2 minutes for 35 cycles. PCR products were run in a 1.5% agarose gel, observed by UV-transluminator and photographed.

RESULTS

From 333 cases of malaria samples, which were collected from Iranian endemic areas during the years 1998-2000, 70.6% were male and 29.4% were female, with a significant difference ($p<0.05$) (Fig 1). In this study, 110 cases (33%) had a history of malaria. The average age of the test group was (22.5 ± 3.1) and infection was more prevalent in the young than the middle-aged and elderly. The distribution of age-related infection showed the highest degree was in the 0-19 year-old group (48.3%) and the lowest in the group over 45 years of age (7.5%) (Fig 2).

By geographical distribution, 48.4% were from Kerman Province (17.1% Sirjan, 31.3% Kahnouj), 34.8% from Sistan-Baluchestan Province (Iranshahr) and 16.8% from Hormozgan Province (Minab) (Fig 3). Distribution of infection was related to occupation, whereas 28.8% of positive cases were laborers and the

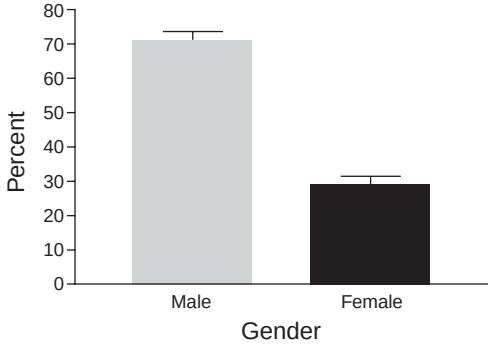

Fig 1- Frequency of malaria cases from endemic areas of Iran by sex.

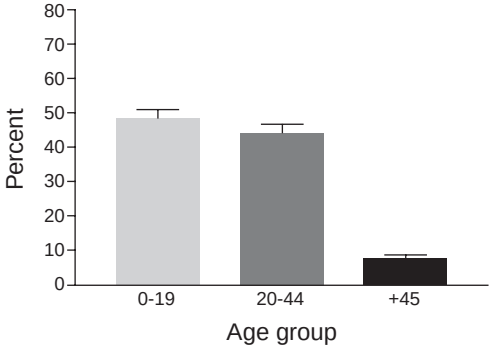

Fig 2- Relative frequency of malaria cases from endemic areas of Iran by age group.

lowest percentage (9.6%) were farmers (Fig 4). There was a relationship between the season and the rate of infection. The highest rate of malaria was observed in autumn (50.5%) and the lowest (2.4%) in winter (Fig 5). Using the microscopic method, 73.6% were found to be *P. vivax*, 28.8% *P. falciparum* and 0.6% a mixed infection of both Plasmodia. However, by molecular assay, 55.9% were *P. vivax*, 15% *P. falciparum* and 29% mixed infections (Figs 6 and 7).

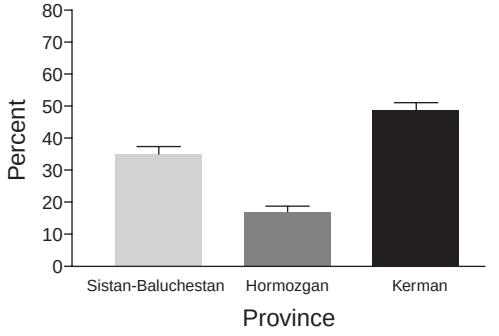

Fig 3- Frequency of malaria cases from endemic areas of Iran by province.

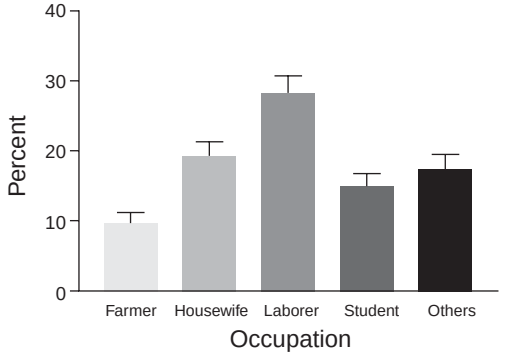

Fig 4- Relative frequency of malaria cases from endemic areas of Iran by occupation.

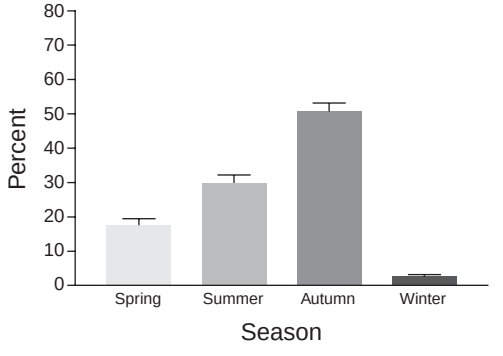

Fig 5- Frequency of malaria cases from endemic areas of Iran by season.

DISCUSSION

The results of this study revealed the incidence rate of malaria was higher in men than women. This might be because in the tropical seasons men work outside the house, and are therefore more exposed to mosquito bites, and the risk of infection is increased. A high rate of infection was found in the young group, indicating the greater sensitivity of this group than elderly people, due to a lack of history of any immune response to Plasmodia. Overall, one third of the cases in this study had a history of malaria and two thirds were infected for the first time. This may highlight a relationship between the history of immune response and the rate of infection. The prevalence rate of malaria was the highest in Kerman Province rather than the other endemic areas, which showed less success in the control and prevention programs. The virulence of Plasmodia depends partly on the strain of parasite and partly on the host. These results showed that the incidence rate alters during malaria epidemiology. The changes depend upon the type of infection, the degree of parasitemia, the strain of Plasmodia, and the method of detection (Nahrevanian and Dascombe, 2001).

Malaria infection seems to be seasonal in endemic areas of Iran and it may be directly related to the activity of anopheline mosquitos. The infection begins in spring (March-April), increases in summer (July-August), peaks in autumn (October-November) and declines in winter (December-January). In the comparison between microscopic and molecular methods in this study, it can be concluded that *P.vivax* was dominant in the endemic areas. Part of this study was confirmed

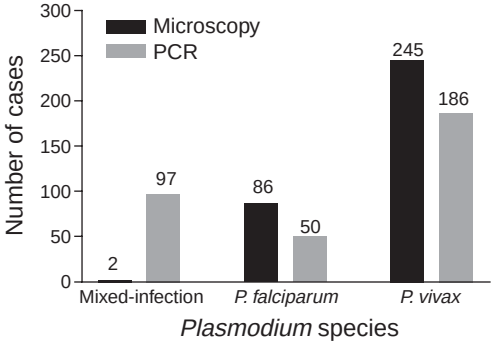

Fig 6- Comparative frequency of microscopic and PCR methods in the diagnosis of *Plasmodium* species.

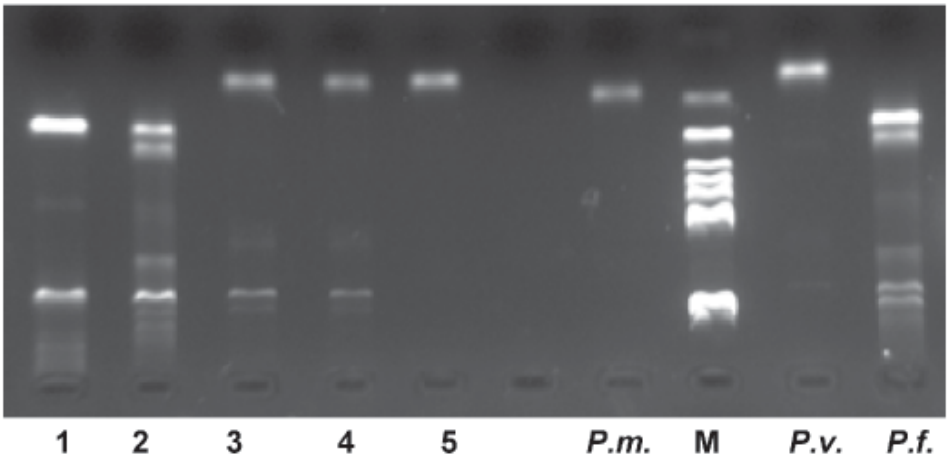

Fig 7- PCR detection using specific primers reacting with prevalent species of human malaria in Iran. (1, 2, 3, 4 and 5 test samples, M marker, Positive controls: *P.m. Plasmodium malariae, P.v. P. vivax* and *P.f. P. falciparum*).

by others in Chahbahar District (Zakeri *et al,* 2002), however, this research gives an overview of mixed malaria infections in all endemic areas of Iran.

In the epidemiological survey by microscopic method, the detection rate of mixed infections was too low (0.6%), when compared with the molecular assay using PCR (29.1%), therefore PCR was more sensitive than microscopic examination. In a direct assay, the microscopist usually focuses on the prevalent species, not the rare cases. The microscopic method was also unable to detect, on some occasions, other forms of *Plasmodium* (*eg* schizonts, merozoites) than the ring form. This might be a reason for *P. falciparum* remaining undetectable in mixed infections. It was concluded that PCR has the advantage of detecting the DNA of Plasmodia of all forms.

Enzymatic amplification of DNA by PCR is a promising method for identifying low levels of parasite DNA. The main limitation of PCR has been the requirement of labor-intensive organic extraction of DNA from whole blood. In addition, the genetic heterogenecity within the Plasmodia requires the development of supplementary molecular procedures to study this parasite. In summary, in a comparative trial between two methods, PCR was sensitive and specific for the detection of Plasmodia. While PCR may ultimately be of value for the diagnosis of malaria, its greatest utility will be in the epidemiological and molecular analysis of Plasmodia infection (Kain *et al,* 1993).

It is concluded that the detection and identification of patients solely by the electrophoretic analysis of PCR products, has proven to be more sensitive and accurate than routine diagnostic microscopy. The PCR assay clearly offers an advantage in this situation. Therefore, the sensitivity of the nested-PCR was greater than the microscopic examination, especially for the detection of mixed-infections ($p<0.05$) in the current study of malaria epidemiology. The detection of mixed infections is important not only for successful medical treatment, but also for finding the true incidence of each species. The PCR technology described provides a powerful tool in the study of malaria biology and epidemiology (Snounou *et al,* 1993a). From the many studies evaluating PCR, it is clear that the technique is more sensitive than microscopy, but the performance is more variable and open to debate (Wilson, 1998).

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