Comparison of light microscopy and nested PCR assay in detecting of malaria mixed species infections in an endemic area of Iran

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Malaria is serious public health problem

Endemic in 104 countries

Malaria caused by obligate intracellular protozoan parasite

Phylum: Apicomplexa

Genus: *Plasmodium*

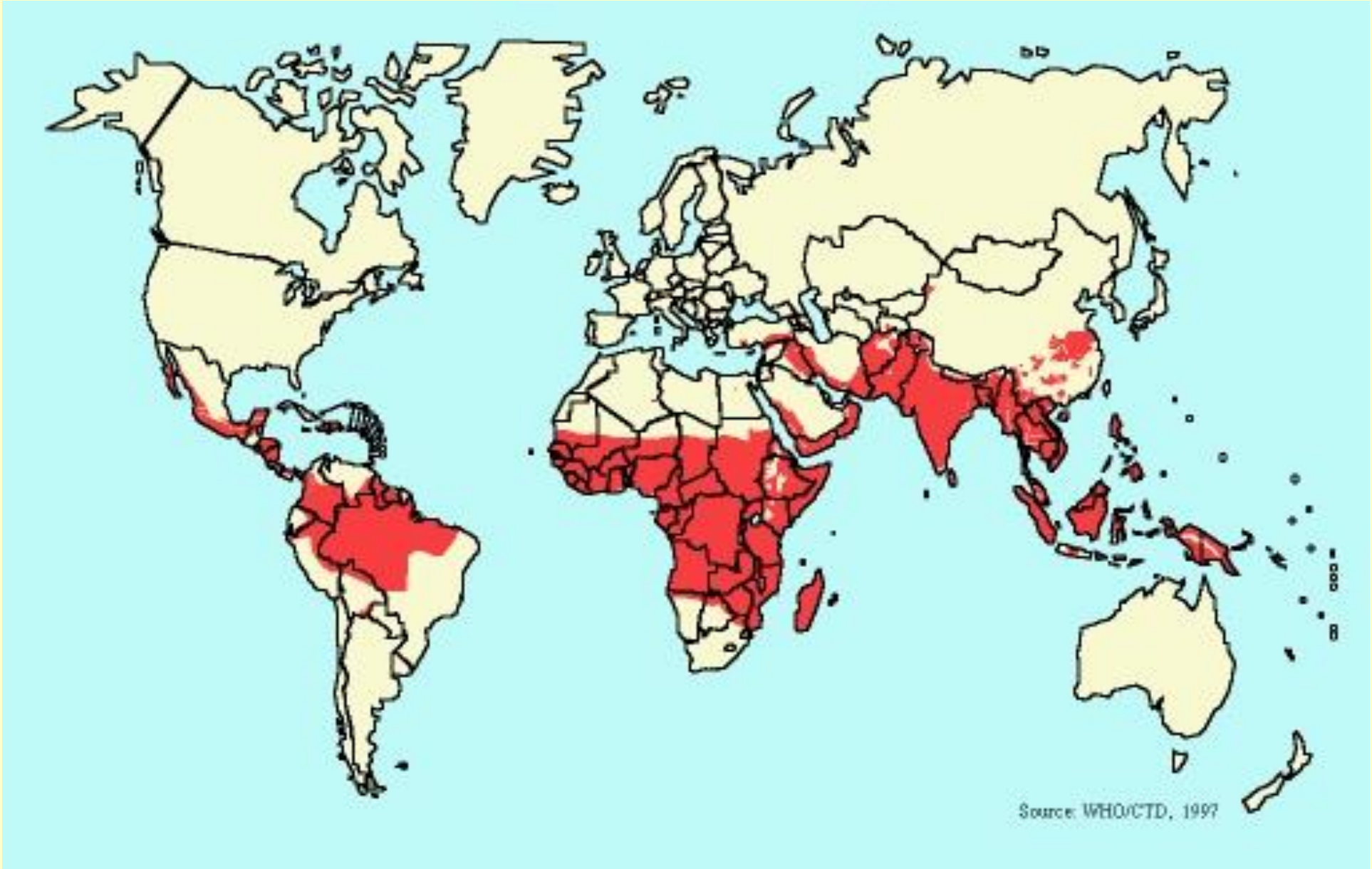
Five Plasmodium species infect human

1. *P. falciparum*
2. *P. vivax*
3. *P. malaria*
4. *P. ovale*
5. *P. knowlesi*

Introduction

- Globally, *Plasmodium falciparum* is the type most responsible for malaria-related mortality,
- *Plasmodium vivax* is the main cause of malaria in Asia, South America and Oceania and is responsible for about 80-90 million cases in the world each year.

Distribution of Malaria



Malaria in Iran

Malaria in Iran

- Most of malaria cases are reported from Sistan –Baluchistan and Hormozgan provinces.
- Malaria transmission in Iran occurs mostly during May-June and October-November.
- Although malaria cases have been decreased in the country in recent years, local malaria transmission continues due to specific reasons, including less socio-economic development and cross-border population movements


IRAN
AZERBAIJAN
TURKMENISTAN
ARMENIA
Tabriz
TURKEY
CASPIAN SEA
Masulé
Gombad-e Kavus
Lake Orumiyeh
Mashhad
IRAQ
Ali-Sadr Caves
Tehran
Hamadan
AFGHANISTAN
Esfahan
Yazd
Chogha Zambil
Kermān
KUWAIT
Shiraz
Persepolis
Zāhedān
Bushehr
Bam
PERSIAN GULF
SAUDI ARABIA
Bandar-e Maqam
Bandar-e Abbās
PAKISTAN
0 200 km
0 120 miles
ARABIAN SEA

➢ WHO Report in Iran, 2010

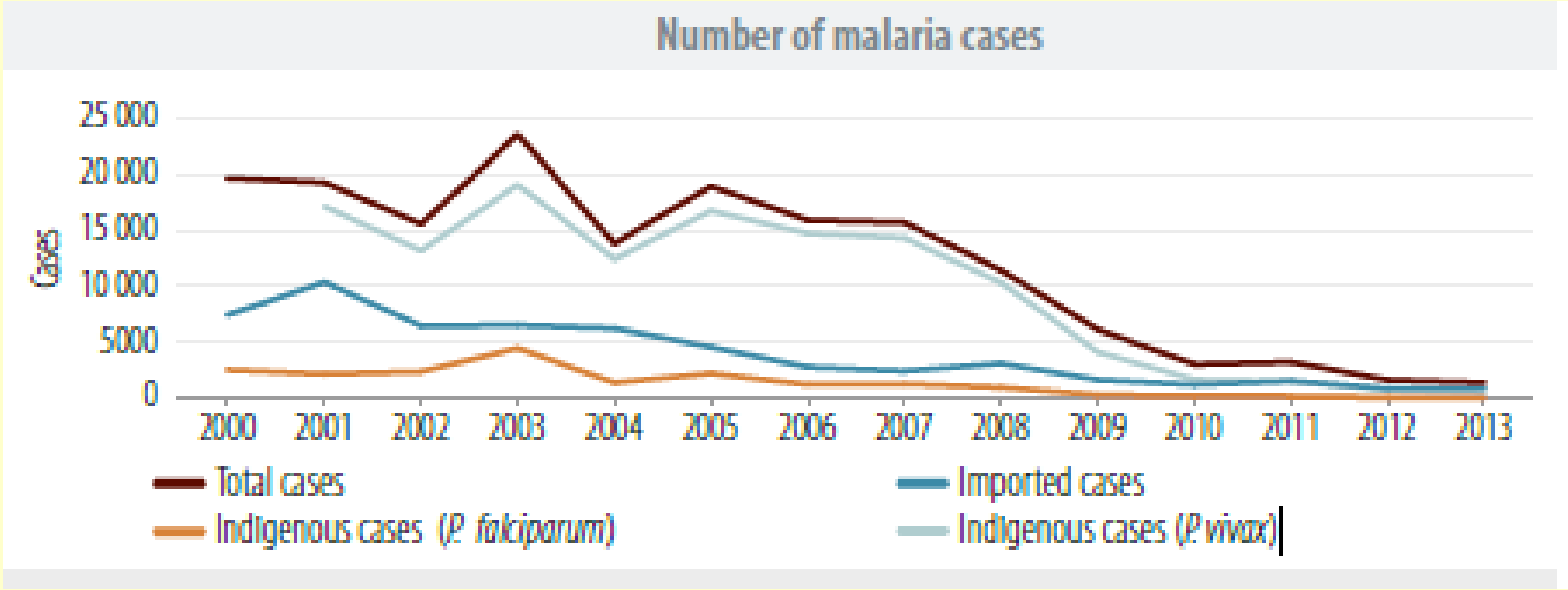
About 16% of Iran population
(12,000,000)

➢8% *P. falciparum*

➢90% *P. vivax*

So dominant species is *P. vivax* in Iran

WHO (Iran Malaria report)



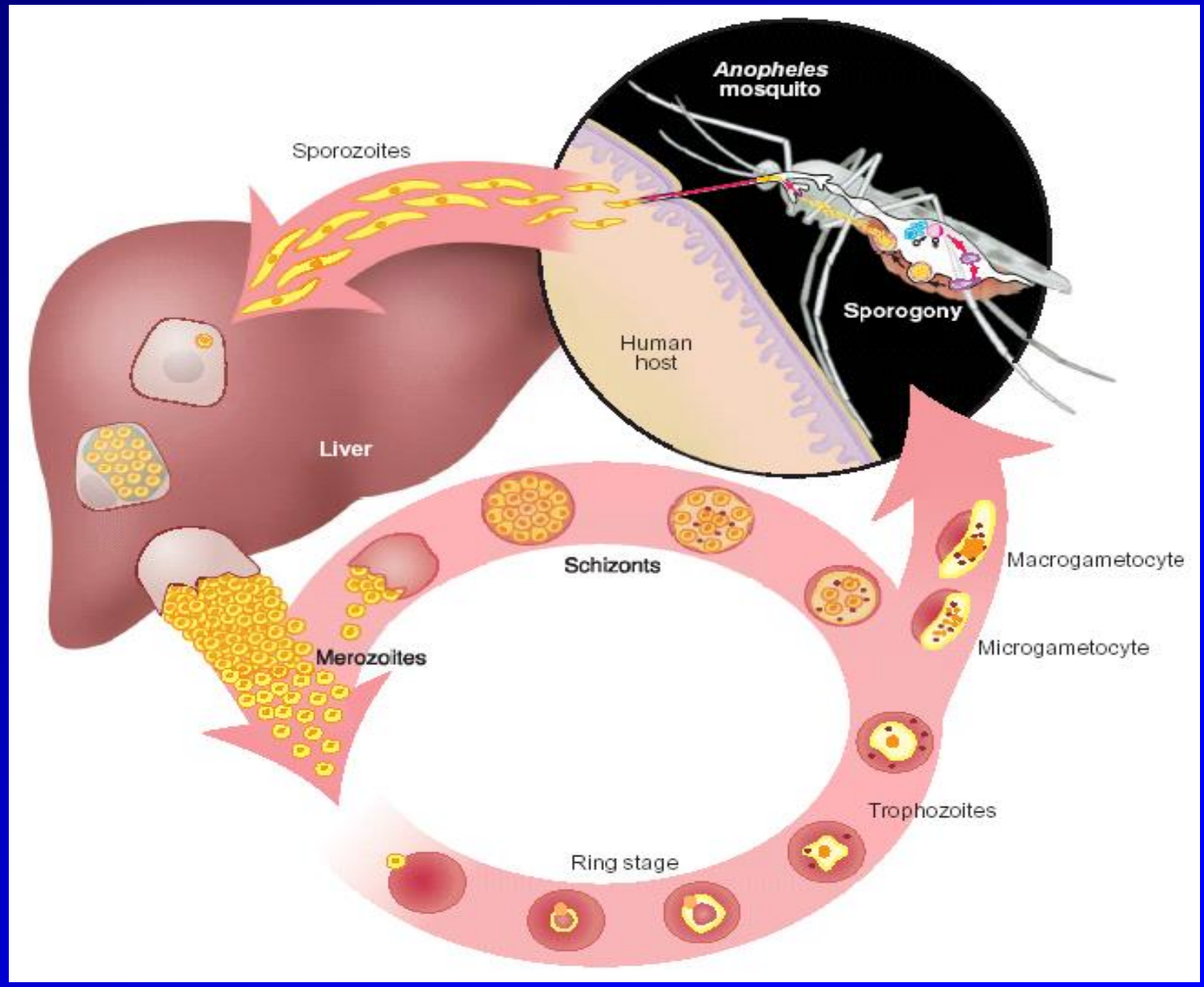
Recent reports in Iran

year	P.falciparum	P.vivax	Mix	Total report
2012	144	1412	67	1623
2013	238	1093	60	1391
2014	113	1114	24	1251
Total	**495 (11.6%)**	**3619 (84.8%)**	**151 (3.5%)**	**4265**

- Both autochthonous and imported malaria have been reported in this area.
- Iran is situated in the Eastern Mediterranean Region
- Southeast of Iran is located in Oriental zone

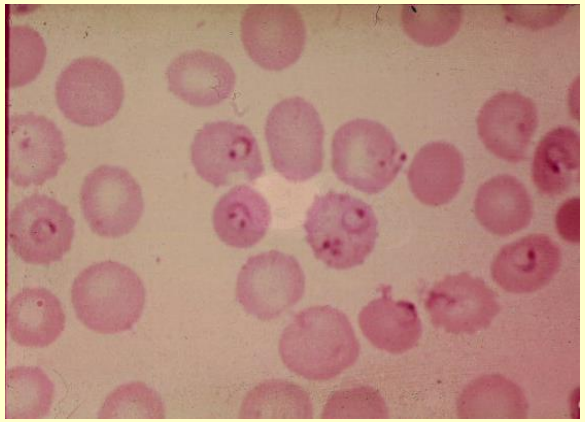
The rest of Iran is situated in Palearctic Zone

Plasmodium Life Cycle



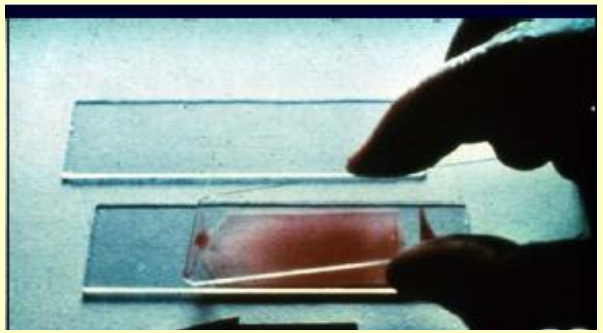
Problem

- Co-existence of two species of the *Plasmodium* genus in a single host (mixed-species infection) has disrupted the diagnosis and treatment of malaria. Furthermore, a delay or failure in detecting *P. falciparum* leads to infection aggravation and rise of the mortality rate, particularly in low or non-immune people. For more precise epidemiological decisions, accurate identification of mixed-species infections is necessary



Light microscopy

- The most common method used for the identification of the species of *Plasmodium* agent of malaria infection is microscopic observation of thick and thin Giemsa-stained blood slides.
- Although this method is simple and inexpensive, its sensitivity decreases in low parasitaemia and mixed-species infections.



PCR

- polymerase chain reaction being the most common method using ribosomal DNA (18s-rRNA).
- Several studies in various parts of the world have revealed the high prevalence of mixed-species infections using PCR. Even in very low parasitaemia, P. vivax and P. falciparum have been detected using this method.

Treatment policy

- At present, *P. falciparum* is resistant to chloroquine and is treated with artemisinin-based combination therapy.
- Chloroquine and primaquine drugs are administered for the treatment of *P. vivax* malaria in the country. Therapeutic decisions on *P. falciparum* and *P. vivax* species are different
- therefore, misdiagnosis of mixed-species of malaria infection may also lead to severe malaria due to failure in the treatment of *P. falciparum* malaria with chloroquine.

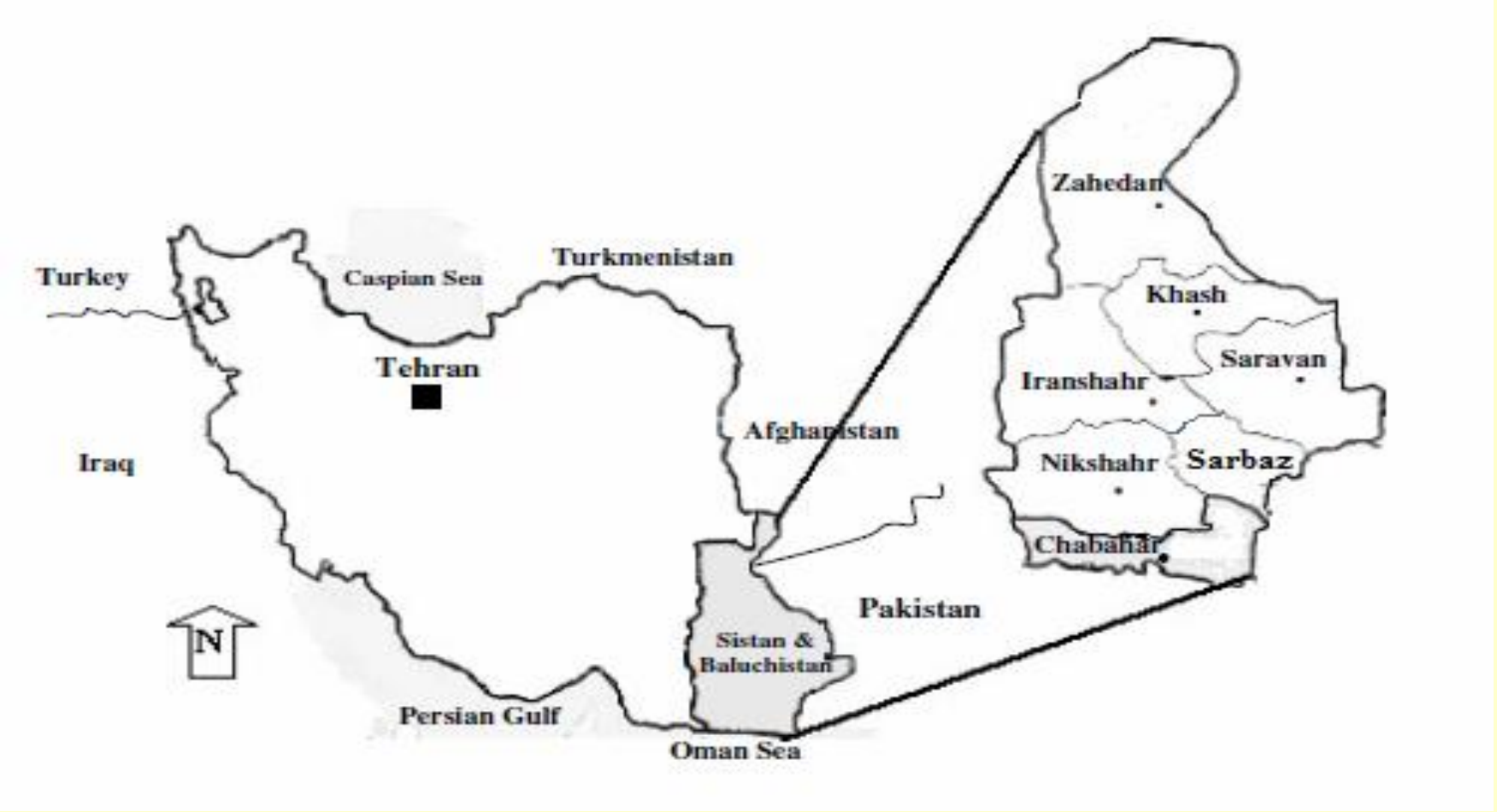
Correct diagnosis

- The accurate detection of mixed-species infections of malaria by more sensitive and specific methods is very critical for successful control programs in Iran due to
- (a) existence of both *P. vivax and P. falciparum* species in endemic areas of Iran
- (b) entering of immigrants with malaria from Afghanistan and Pakistan (countries with 39000 and 282000 malaria cases in 2013 respectively)
- (c) insecticide resistance and the presence of drug-resistant strains in the region.

MATERIALS AND METHODS

- Study sampling was performed between May-November 2013.
- A total of 160 malaria patients were included out of 205 randomly suspected symptomatic malaria patients using microscopy.
- Blood samples were obtained from individuals who were clinically suspected for malaria (having symptoms, such as chills, fever and sweating) and attended health care centers in Pishin (5 km from Pakistan borders) and Yahocalat in Sarbaz and Chabahar districts in Sistan and Baluchestan province.
- Thick and thin blood smears were stained with 3% Giemsa and examined microscopically by two blinded independent examiners.

Study area


Turkey
Caspian Sea
Turkmenistan
Tehran
Iraq
Afghanistan
Zahedan
Khash
Saravan
Iranshahr
Nikshahr
Sarbaz
Chabahar
Pakistan
Sistan &
Baluchistan
N
Persian Gulf
Oman Sea

Microscopy observation

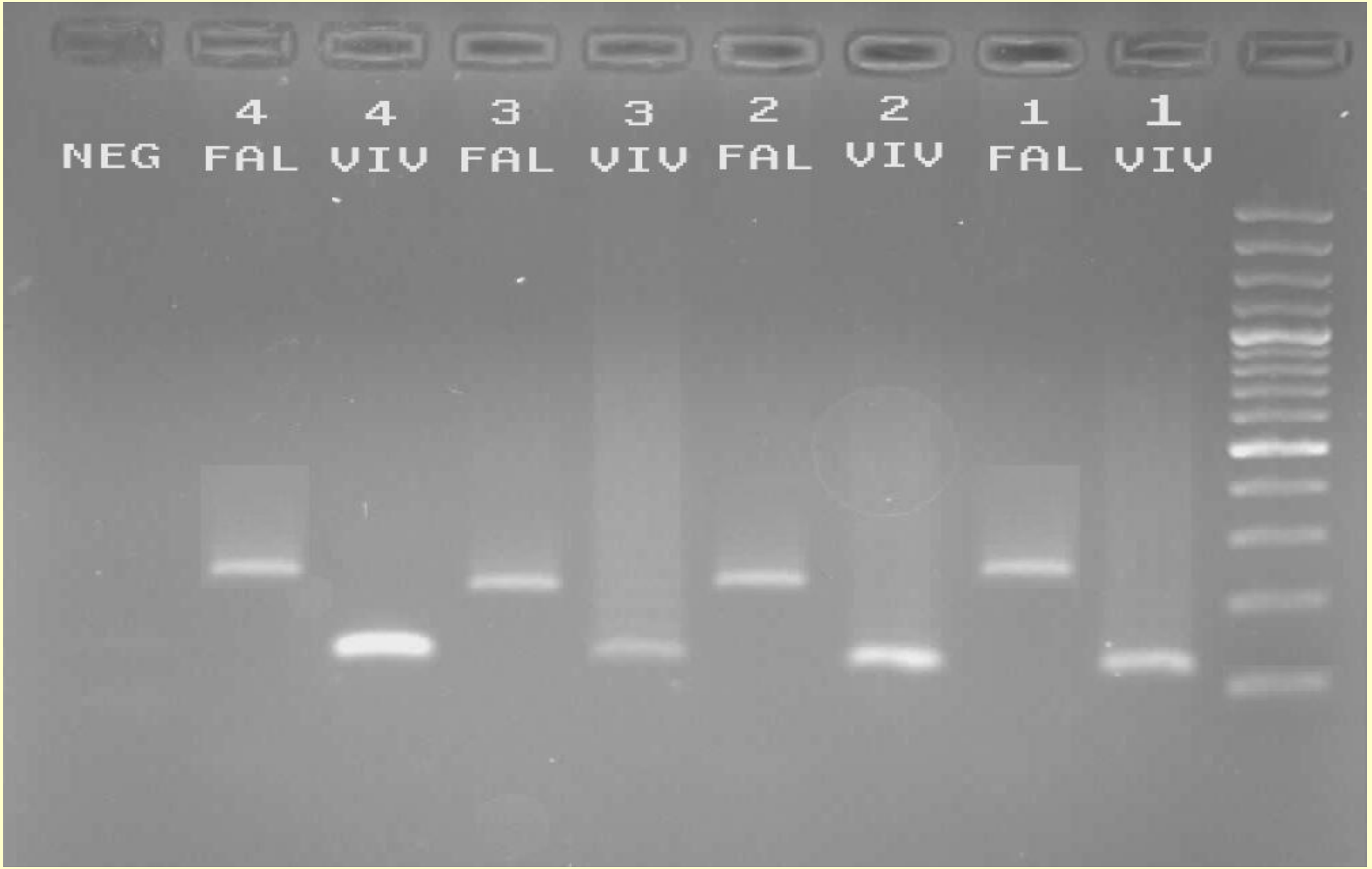
- At least 200 thick and thin blood fields at 1000× magnification were examined for Plasmodium detection and species differentiation, respectively. Parasite density in thick blood films was calculated as numbers of asexual parasites per 200 white blood cells.
- Whole blood samples were kept in EDTA-coated tubes and the samples were frozen for later PCR analysis.

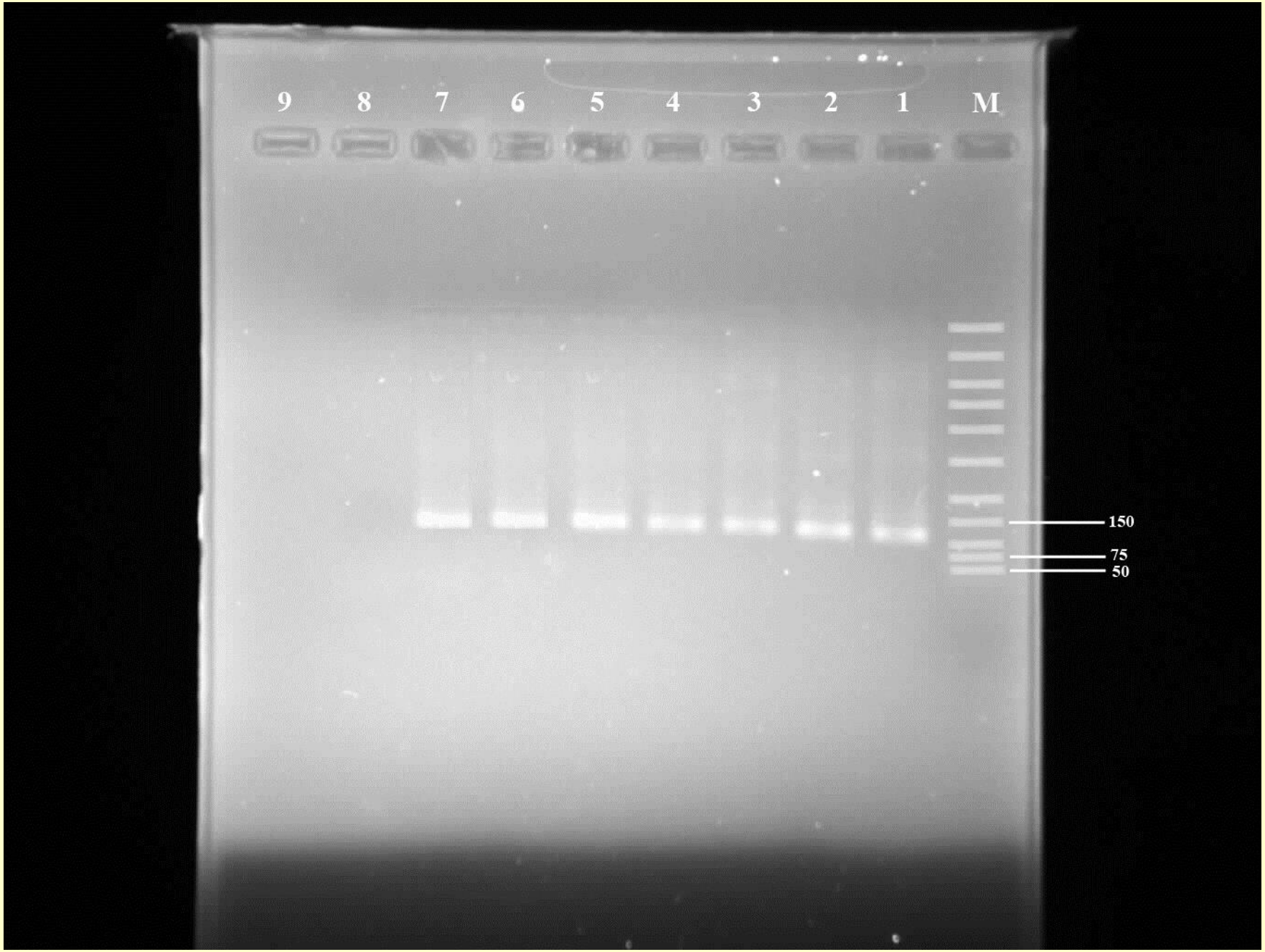
Nested PCR

- DNA was derived from 200 µL blood samples using a QIAquick PCR purification kit (Qiagen, Germany), according to the manufacturer's instruction.
- DNA template was eluted in 200 µL of double-distilled water, and approximately 100 ng of DNA template was used in each PCR assay. Nested PCR was performed using the Plasmodium 18 subunit ribosomal ribonucleic (Ssr RNA) genes to detect the mixed-strain infections

Electrophoresis

- The amplified products were run in 2% agarose gel electrophoresis and stained by DNA Safe Stain for visual detection of bands using ultraviolet transillumination.


NEG
4 FAL
4 VIV
3 FAL
3 VIV
2 FAL
2 VIV
1 FAL
1 VIV


9
8
7
6
5
4
3
2
1
M
150
75
50

Results

- Patients ages ranged from 4 to 58 years, with an average of 30 years. Eighty-seven percent of patients were males.
- Parasitemia ranged from 120 to 100000 parasites per µl of blood with mean 9500 p/µl. The burden of parasitaemia in ten patients was below 1000 parasites/µL.
- In total, 1.875% (3 patients) and 11.25% (18) of patients indicated mixed species infections in microscopy and nested PCR, respectively
- The sensitivity of light microscopy for detection of mixed species malaria infections was 16.6% (95% CI 3-49.1).
- There was a significant difference between sensitivity of microscopy and nested PCR in identifying mixed species malaria infections ($P<0.0009$).

Table 1: The results of malaria species detection in patients with clinical malaria according to two methods in southeastern of Iran.

		Nested PCR			
		P. falciparum N=30	*P. vivax* N=112	Mixed *Pf/Pv* N=18	Total N=160
Microscopy	*P. falciparum*	**30**	0	**2**	32
	P. vivax	0	**112**	**13**	125
	Mixed *Pf/Pv*	0	0	**3**	3
	Negative	0	0	0	0

Discussion

- It seems in mixed-species infections, one species often dominates the other numerically.
- Therefore, identifying the two species in such infections could be complicated by conventional light microscopy

The mixed-species infections detected by microscopy were above 2000 P/µl and the sensitivity of microscopy was decreased with declining parasitemia.

Conclusion

- Our findings show that nested PCR assay can facilitate the better diagnosis of mixed species infections and the correct treatment of malaria patients especially in regions where prevalence of *Plasmodium falciparum* resistant to chloroquine is high.
- It is also concluded that mixed species infection is almost common in the region where both species coexist and their detection is only based on traditional microscopy that may underestimate their importance.