APPLICATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETERMINATION OF THE HUMAN BLOOD INDEX IN ANOPHELINE MOSQUITOES COLLECTED IN IRAN[1]

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ABSTRACT. The microplate method of an ELISA was modified for identification of human blood meals from 5,325 engorged mosquitoes belonging to 12 species of *Anopheles* captured in 19 provinces of Iran. Four hundred and four (7.5%) specimens reacted with the ELISA anti-human alkaline phosphatase conjugate. The human blood index in nine species of *Anopheles* varied from 3.6 to 23.7%. The results of this field application of the ELISA indicated that the technique is practical, reproducible and generally a suitable serological test for determination of human blood index of the anopheline mosquitoes.

INTRODUCTION

"The human blood index" or the proportion of *Anopheles* giving a positive reaction for human blood is a valuable indicator of the importance of an *Anopheles* species to serve as a vector of human malaria (Bruce-Chwatt 1980). Different techniques, most commonly the serological precipitin tests, have been employed for determination of the human blood index in anopheline mosquitoes and other blood sucking arthropods. Informative reviews of the techniques applied have been prepared by Weitz (1956) and Tempelis (1975).

The microplate method of the enzyme-linked immunosorbent assay (ELISA) described by Voller et al. (1974) was first modified for identification of *Anopheles* bloodmeals in an experimental study on *An. stephensi* Liston fed on human volunteers and guinea pigs with very good results by Edrissian and Hafizi (1980).

The ELISA has been also applied by Burkot et al. (1981) in identification of the bloodmeals of laboratory mosquitoes fed on several host animals with identification of blood sources to the generic level.

In this study the modified microplate method of the ELISA (Edrissian and Hafizi 1982a) has been applied in the field for determination of the human blood index in the *Anopheles* species collected from different parts of Iran.

MATERIALS AND METHODS

BLOODMEALS. Collections of blood-fed mosquitoes were made by malaria entomologists and field technicians of Malaria Eradication Units and the Institute of Public Health Research in 19 provinces of Iran during 1982–84. Species of *Anopheles* were captured in the rural areas by suction tube or spray catching methods inside human dwellings, stables, storerooms and outdoor resting places around villages.

The bloodmeals of the identified *Anopheles* were smeared on Whatman filter paper and dried. They were interleaved with non-absorbent "onionskin" paper and packed inside plastic bags and sent with the necessary information to the Protozoology Unit, Department of Medical Parasitology and Mycology in Teheran for ELISA testing. Collected samples were stored in a desiccator, containing calcium chloride, at room temperature before testing.

ELISA PROCEDURE. The microplate method of ELISA described by Voller et al. (1974) and modified for identification of *Anopheles* bloodmeals by Edrissian and Hafizi (1982a) was employed as follows:

The dried spots of blood were cut out of small paper discs, 2–3 mm in diameter. Each disc was put in a well of the Micro ELISA plate (Dynatech Laboratories, Inc.). Elution of dried blood from the filter paper was done with 50 μl of distilled water added to each well and left for 2 hr inside a humid box (enamel covered pan) at room temperature.

Then 50 μl of coating buffer (carbonate bicarbonate buffer, pH 9.6) was added to each well. The discs of filter paper were stirred inside the wells and removed with a small dissecting needle.

The needle was rinsed in a small jar of distilled water and dried with a clear piece of cotton wool after each use. For coating to proceed, the plates were left overnight at +4°C inside the humid box.

Next morning the plates were washed with phosphate buffered saline (PBS)—Tween 20(pH 7.2) three times, each time for 3 min.

The goat anti-human IgG conjugated to alkaline phosphatase (Miles Laboratories, Ltd.

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and supplied through the World Health Organization) was diluted to 1:1000 (in one batch) and 1:500 (in another batch) in PBS—Tween and 100 μl was added to each well. The plates were then incubated for 2 hr in a humid box at room temperature, removed and washed as before.

Then 100 μl of substrate solution (1 mg/ml P-nitrophenyl phosphate, Sigma 104, in 10% diethanolamine buffer pH 9.8 containing 0.5 m mol/liter $MgCl_2$ and 0.02% NaN_3) was added to each well.

Two wells without blood (blanks), two wells with mouse blood (negative controls) and two wells with human blood (positive controls) were used in each plate. The amount of the blood sample in each positive and negative control was approximately 1 μl which were put on filter paper, dried, packed and stored as the test samples.

Results were assessed subjectively by examination with the naked eye and the yellow color, which indicated a positive reaction, this usually starting to appear in 10 min.

The end-point results were read in most tests 30–60 min after addition of substrate solution. Positive controls (the wells of human blood samples) and negative controls were assigned values of ++++ and 0, respectively. The test samples were estimated from 0 to ++++ according to the strength of the yellow color produced in each well. The end-point results were read before the blanks and negative controls clearly became yellow. However, the reaction could be stopped by addition of 10 μl of NaOH solution (3 mol/liter) after 30 min or any appropriate period of time, depending on different factors such as conjugate and room temperature.

RESULTS AND DISCUSSION

In the ELISA testing of 5,325 bloodmeals collected from 12 species of *Anopheles* captured from 19 provinces of Iran, 404 (7.5%) specimens showed positive reactions from + to ++++ with alkaline phosphatase anti-human conjugate. The results for each species of *Anopheles*, in relation to their resting places, are given in Table 1. Number of engorged anopheline mosquitoes collected in each province, their human blood index and also the mean malaria parasite incidence during 8 months of the year (starting from March 21) during 3 years of collection of anopheline mosquitoes (1982–84) are given for each province in Table 2.

The human blood index for nine anopheline species in which the number of individuals tested was over 100, ranged from 3.6 to 23.7% (Table 2). As expected, it was highest in mosquitoes collected from human dwellings (Table 1).

The obtained data may not indicate the exact true human blood index in the species of *Anopheles* in the studied areas, because sampling was not designed for such a purpose, and the collection of anopheline mosquitoes was carried out whenever it was possible. In the southern provinces of Iran such as Hormozgan and Sistan and Baluchestan provinces, where the incidence of malaria parasites is relatively high, there are more anopheline species in the collected samples. But there is no relation between the ELISA positivity rate and the malaria parasite incidence (Table 2).

All the bloodmeals were tested during the 6 months after collection. The ELISA positivity rate and strength of the bloodmeal samples

Table 1. Human blood index determined by ELISA in the *Anopheles* species collected in different resting places in Iran during 1982–84.

Species	Human dwelling		Stable		Storeroom		Outdoor		Not recorded		Total		
	No.	ELISA pos.	No.	ELISA pos.	No.	ELISA pos.	No.	ELISA pos.	No.	ELISA pos.	No.	ELISA pos.	% pos.
An. culicifacies	2	0	93	0	—	—	—	—	456	26	551	26	4.7
An. dthali	211	12	194	39	37	7	—	—	75	7	517	65	12.5
An. fluviatilis	1	0	112	0	1	1	—	—	21	6	135	7	5.1
An. hyrcanus	7	0	37	0	1	0	—	—	—	—	45	0	0.0
An. maculipennis	121	19	657	33	226	6	20	2	201	1	1225	61	4.9
An. multicolor	84	22	17	2	—	—	—	—	—	—	101	24	23.7
An. pulcherrimus	81	2	15	0	3	2	—	—	52	11	151	15	9.9
An. sacharovi	49	13	212	20	—	—	—	—	8	0	269	33	12.2
An. sergentii	—	—	3	0	—	—	—	—	—	—	3	0	0.0
An. stephensi	2	1	1160	40	1	0	—	—	29	3	1192	44	3.6
An. superpictus	146	27	805	96	27	2	112	1	27	2	1117	128	11.4
An. turkhudi	1	0	16	0	—	—	—	—	2	1	19	1	5.2
Total	705	96	3321	230	296	18	132	3	871	57	5325	404	7.5
% ELISA positive		13.6		6.9		6.0		2.2		6.5		7.5	

Table 2. Collected engorged *Anopheles* species from different provinces of Iran during the years 1982–84, their human blood index (% ELISA positive) and the malaria parasite incidence of each province.

	Provinces from north to south																				
Anopheles species	E. Azarbaijan	W. Azarbaijan	Gilan	Mazandaran	Teheran	Semnan	Kurdistan	Hamadan	Bakhtaran	Loristan	Esfahan	Chahar-Mahal & Bakhtiary	Khorasan	Khusistan	Kohkiluyeh & Boir-Ahmad	Boushehr	Kerman	Hormozgan	Sistan and Baluchestan	Total	% ELISA pos.
*An. sacharovi**	254	—	—	—	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	269	12.2
*An. maculipennis**	—	32	156	588	3	15	79	—	—	—	161	144	16	—	31	—	—	—	—	1225	4.9
An. hyrcanus	—	—	—	45	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	45	0.0
*An. superpictus**	—	—	—	—	—	16	103	49	30	6	58	112	650	73	2	—	2	16	—	1117	11.4
An. multicolor	—	—	—	—	—	—	—	—	—	—	—	—	101	—	—	—	—	—	—	101	23.7
An. pulcherrimus	—	—	—	—	—	—	—	—	—	—	—	—	—	132	—	4	—	1	14	151	9.9
An. sergentii	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2	—	3	0.0
*An. dthali**	—	—	—	—	—	—	—	—	—	—	—	—	—	278	—	159	—	20	60	517	12.5
*An. fluviatilis**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	8	104	22	135	5.1
*An. stephensi**	—	—	—	—	—	—	—	—	—	—	—	—	—	9	1	1	5	1176	—	1192	3.6
An. turkhudi	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	9	10	19	5.2
*An. culicifacies**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	551	551	4.7
Total	254	32	156	633	3	31	182	49	30	6	219	256	767	507	34	165	16	1328	657	5325	7.5
% ELISA positive	12.2	50.0	2.5	1.7	0.0	38.7	5.4	0.0	46.6	0.0	1.3	0.3	17.6	2.3	8.8	33.9	0.0	4.2	5.9	7.5	—
MMPI/1000 POP**	0.020	0.008	0.011	0.030	0.055	0.260	0.327	0.017	0.167	2.153	0.180	1.519	0.211	0.744	2.970	0.853	1.762	9.242	18.923	0.850	

* Known malaria vectors in Iran.

** The mean of the malaria parasite incidence/1000 population/8 months (from 21 March) in the years 1982–84.

collected in 1982 were generally higher than the related findings of the samples collected in 1983–84 (The ELISA positivity rates were 10.5 and 3.7 in 1982 and 1983–84, respectively.) This difference may be due to the variations in the conditions and sites of sampling, preparation of the bloodmeals, length of the storage period, the strength of the ELISA conjugate and the ELISA testing conditions, as well as some other unknown factors related to the biology of the *Anopheles* species and their environments.

There was very little difference between the ELISA results of the human dried bloodstains tested freshly or after storing in a desiccator at room temperature from 2 to 6 weeks (Edrissian and Hafizi 1982b). Therefore, the collected bloodmeals for ELISA test could be stored inside a desiccator jar at room temperature at least 6 weeks and most probably for a much longer period of time in a refrigerator as it has been reported in the case of the precipitin test (World Health Organization 1975).

The results of this field application of the modified microplate method of ELISA to determine human blood index in the *Anopheles* species, as well as the results of the previous experimental application of this serological method (Edrissian and Hafizi 1982a) showed that the ELISA is a simple technique to perform and the end-point result is quite easy to read. A trained and experienced technician could easily test about 1,000 samples per week.

A comparison of the ELISA with the slide gel diffusion precipitin test showed that the ELISA is more sensitive and even more specific in detection of human blood stains (Edrissian and Hafizi 1982b). However, the gel diffusion technique has been reported a suitable (Crans 1969), rapid, uncomplicated and an inexpensive way to determine locally the origin of mosquito bloodmeals (Collins et al. 1983).

Nevertheless, as the essential materials of the ELISA test including conjugate are commercially available, it is quite practical to apply this technique in a simple serological laboratory for examination of bloodmeals of anopheline mosquitoes and most probably other blood-sucking arthropods in most countries.

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References Cited

Bruce-Chwatt, L. J. 1980. Essential malariology. 1st ed. William Heinemann Medical Books Ltd., London.

Burkot, T. R., W. G. Goodman and G. R. DeFoliart 1981. Identification of mosquito bloodmeals by enzyme-linked immunosorbent assay. Am. J. Trop. Med. Hyg. 30:1336–1341.

Collins, R. T., B. K. Dash, R. S. Agarwala and K. B. Dhal. 1983. An adaptation of the gel diffusion technique for identifying the sources of mosquito bloodmeals. WHO/MAL/83.992,WHO/VBC/83.875.

Crans, W. J. 1969. An agar gel diffusion method for the identification of mosquito bloodmeals. Mosq. News 29:563–566.

Edrissian Gh.H. and A. Hafizi. 1980. Application of enzyme-linked immunosorbent assay (ELISA) in identification of *Anopheles* bloodmeals. Abstracts. 10th Int. Congr. Trop. Med. Malaria, Manila, November 9–15, 1980, p. 81.

Edrissian Gh.H. and A. Hafizi. 1982a. Application of enzyme-linked immunosorbent assay (ELISA) to identification of *Anopheles* mosquito bloodmeals. Trans. R. Soc. Trop. Med. Hyg. 76:54–56.

Edrissian Gh.H. and A. Hafizi. 1982b. Detection of human bloodstains by enzyme-linked immunosorbent assay (ELISA). Acta Med. Iran. 24:57–69.

Tempelis, C. H. 1975. Host-feeding patterns of mosquitoes, with a review of advances in analysis of bloodmeals by serology. J. Med. Entomol. 6:635–653.

Voller, A., D. Bidwell, G. Huldt and E. Engvall. 1974. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bull. W.H.O. 51:209–211.

Weitz B. 1956. Identification of bloodmeals of blood sucking arthropods. Bull. W.H.O. 15:473–490.

World Health Organization. 1975. Manual on practical entomology in malaria, part II. Methods and techniques, pp. 127–135. WHO Offset Publ. 13, Geneva.