APPLICATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETERMINATION OF THE HUMAN BLOOD INDEX IN ANOPHELINE MOSQUITOES COLLECTED IN IRAN1

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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 of 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 50g.1 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. For coating to proceed, the plates were left overnight at 4°C inside the humid box (enamel covered pan) at room temperature.

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.
and supplied through the World Health Organ-
ization) 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 be-
fore.

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 MgCl₂ and 0.02% NaN₃) 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 exami-
nation 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 ++++ ac-
cording to the strength of the yellow color pro-
duced 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 ap-
propriate period of time, depending on dif-
ferent factors such as conjugate and room tem-
perature.

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%) speci-
mens 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 prov-
ince, 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 mos-
quitos (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
ture 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 Sis-
tan and Baluchestan provinces, where the inci-
dence of malaria parasites is relatively high,
there are more anopheline species in the col-
llected samples. But there is no relation between
the ELISA positivity rate and the malaria para-
site incidence (Table 2).

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

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Human dwelling</th>
<th>Stable</th>
<th>Storeroom</th>
<th>Outdoor</th>
<th>Not recorded</th>
<th>Total</th>
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<td>ELISA No.</td>
<td>ELISA No.</td>
<td>ELISA No.</td>
<td>ELISA No.</td>
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<td>96</td>
<td>3521</td>
<td>230</td>
<td>296</td>
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% ELISA positive | 15.6 | 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.

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<th>Anopheles species</th>
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<th>W. Azerbaijan</th>
<th>Gilan</th>
<th>Mazandaran</th>
<th>Tehran</th>
<th>Semnan</th>
<th>Kurdistan</th>
<th>Hamadan</th>
<th>Bakhtar</th>
<th>Loristan</th>
<th>Esfahan</th>
<th>Chahar-Manush &amp; Bakhtiary</th>
<th>Khorasan</th>
<th>Khuzistan</th>
<th>Koohkulal &amp; Bor-Ahmad</th>
<th>Boushehr</th>
<th>Kerman</th>
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</table>

% 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.

ACKNOWLEDGMENTS

The authors would like to thank the malarialogists, entomologists and field technicians of Malaria Eradication Units and the Institute of Public Health Research for their cooperation in collection and identification of anopheline mosquitoes and preparation of bloodmeal samples. We wish to acknowledge also the help of Miss Sh. Ansari and Mr. A. Kannani in testing of the bloodmeal samples collected in 1983 and 1984.

References Cited


