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DEVELOPMENT OF NEW TECHNIQUES FOR THE IDENTIFICATION OF HOST BLOOD MEALS IN ARTHROPOD VECTORS

REPORT OF THE INTERLABORATORY TRIAL 1986-1987

Ecology and Control of Vectors Unit
Division of Vector Biology and Control

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1. INTRODUCTION

The identification of blood meals in arthropod vectors has served as an important tool for ecological investigations, for epidemiological surveillance and for strategies to control various human vector-borne infections.

Apart from biochemical and other approaches, several immunological techniques were introduced for this purpose as reviewed by Weitz (1956), Tempelis (1975) and Washino & Tempelis (1983). However, many of these tests were difficult to perform, not sufficiently sensitive and/or specific, expensive, and conducted only at certain expert centres. The recent development in technology based on immunological principles has offered a whole spectrum of new tests for this purpose. These were reviewed at a meeting of investigators on the development of new techniques for the identification of host blood meals in arthropod vectors, sponsored and organized jointly by the Division of Vector Biology and Control, Ecology and Control of Vectors Unit, and the WHO Microbiology and Immunology Services[1] and held at the WHO Immunology Research and Training Centre (IRTC) in Geneva, 1-3 October 1985. During the discussion at that meeting, it became obvious that:

(a) the new immunological techniques based largely on solid phase assay systems (such as dot blot and dipstick enzyme immunoassays) could be expected to contribute to the development of simpler, rapid, more sensitive, and inexpensive blood meal tests over the next few years;

(b) the use of improved reagents (polyvalent antibodies absorbed in solid phase and/or monoclonal antibodies) would improve the specificity and permit the standardization of the tests; and

(c) the simplicity and economy may be achieved by development of test kits which would make it possible "to perform the tests at regional, national and individual levels, where field entomologists could gain rapid access to information."

As a result of that meeting, the participants proposed a closer collaboration among the interested parties for the development of blood meal assay kits to be coordinated by the WHO IRTC, Geneva, with the following priorities:

(a) to assess the methods currently available or developed by the participating laboratories in an interlaboratory trial;

(b) to select suitable assay(s) and to formulate kits initially designed to provide human/non-human discrimination with subsequent identification of a range of species if required;

(c) to test these kits in selected endemic areas; and

(d) to establish the quality control of the test kits in conjunction with reference centre(s) to be created.

This report describes the organization and the results of an interlaboratory trial, organized by the WHO IRTC during the period 1986-1987.

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1 Formerly Immunology Unit, World Health Organization, Geneva.

2. MATERIALS

2.1 Specimens of mosquitos

The specimens of mosquitos (Anopheles stephensi) prepared by Dr B. Betschart, Swiss Tropical Institute, Basel, Switzerland, were fed through a membrane as follows: fresh human blood (3 ml with EDTA as anticoagulant) was placed on a ribbled aluminium plate and covered by stretching parafilm over the plate. The blood kept at a temperature of 37°C was then offered to the mosquitos through the net of the cage. The mosquitos which had taken a blood meal were sorted out at 4°C and killed by squashing of the immobilized insects on Whatman No. 1 filter paper, at different time intervals after feeding. Each group consisted of 50 mosquitos. The first group was killed immediately after feeding (Code No. 1), other groups at 8, 24, 32 and 48 h after feeding, respectively (Code Nos. 2, 3, 4 and 5). Another group of mosquitos was fed on glucose only (Code No. 6). The specimens were kept under desiccation (silica gel) at -20°C following collection, sent to the WHO IRTC on dry ice and stored in Geneva at -20°C until dispatch.

2.2 Specimens of whole blood

The specimens of whole blood were prepared from different species by WHO IRTC as follows: cow (Code No. 7), man (Code Nos. 8 and 12), dog (Code No. 9), pig (Code No. 11), goat (Code No. 13) and chicken (Code No. 15). In addition, two mixtures were prepared by mixing human blood with bovine blood in ratios 1:10 (Code No. 10) and 1:100 (Code No. 14), respectively. No anticoagulants were used except for mixtures (Code Nos. 10 and 14) in which human heparinized blood was used in order to prevent clotting. All specimens, obtained by venipuncture, were immediately spotted on Whatman No. 1 filter paper in 50 µl aliquots and left to dry at room temperature followed by incubation at 37°C for 1 h. Then the specimens were kept in sealed plastic bags containing silica gel at -20°C.

When the collection of specimens was completed, the samples were coded as indicated above, distributed into plastic-lined foil bags containing silica gel and dispatched by mail to the participants. Each participant received:

(a) 3 specimens of individual mosquitos killed at different times after feeding on human blood (Code Nos. 1-5) and/or fed on glucose only (Code No. 6);

(b) one specimen of blood from different species and/or mixtures of blood (Code Nos. 7-15);

(c) a standard paper punch (1/4" Round Die, No. 403R, McGill Metal Products Co., Marengo, Illinois, USA); and

(d) instructions for testing asking the participants:

  (i) to use the punch for all samples in an attempt to standardize the size of filter paper discs used for elution; it was assumed that the discs taken in the middle of the blood specimens contained about 10 µl of blood;

  (ii) to elute the discs with an appropriate diluent according to the tests used (composition, volume and pH of the diluent to be recorded, the elution to be carried out at 20°C for 2 h);

  (iii) to test the samples in duplicate by technique(s) used in their laboratory for distinguishing between human and non-human sources of blood;

  (iv) the samples identified as human to be titrated to the end-points using 10-fold dilutions; and

  (v) to report the detailed results including the description of reagents and technical details to the WHO IRTC for compilation.

3. METHODS

All participants received the consignments sent by mail in good order, except one, delivered broken with some samples and punch missing; these were replaced by an identical set on request.

All participants used the punch for preparation of discs and performed the elution of samples at room temperature for 2 h as requested. Most of them titrated the samples identified as human to the end-points using 10-fold dilutions (exceptions are described in the text). The tests performed were as follows:

3.1 Complement fixation test (CFT) using polyvalent antibody to human immunoglobulin G (IgG) and/or to the IgG of other species and double dilutions of the test samples to the end-point (Staak et al., 1981).

3.2 Reverse passive haemagglutination assay (RPHA) using glutaraldehyde-fixed sheep red blood cells coated with polyvalent antibodies to human IgG or albumin and to immunoglobulins (Ig) of other species.

3.3 Double gel diffusion (DGD) using polyvalent antibodies to human serum proteins and/or to serum proteins of other species (Collins et al., 1986).

3.4 Direct enzyme-linked immunosorbent assay (ELISA) using eluted blood samples as the coating antigens, horseradish peroxidase (HRP) conjugated polyvalent antibody to human Ig and relevant substrate with end-points read by ELISA reader.

3.5 ELISA sandwich (ELISA-S) using monoclonal antibodies to human IgG for both the capture and the revealing antibodies (the latter being labelled with HRP), results read with naked eye (Service et al., 1986); double dilutions of the test samples to the end-point.

3.6 ELISA inhibition (ELISA-I) using polyclonal (ELISA-I-p) and monoclonal (ELISA-I-m) antibodies to human IgG or to the IgG of other species as capture layer, HRP-labelled homologous IgG which competes with the test sample and relevant substrate with end-points read by ELISA reader.

3.7 ELISA dot blot (ELISA-DB) using nitrocellulose filter paper in different modifications: ordinary ELISA with HRP-labelled polyclonal (ELISA-DB-p) antibody (Roy & Sharma, 1987), ELISA with double polyvalent antibody using peroxidase-antiperoxidase soluble complexes (ELISA-DB-PAP) as detectors (Lombardi & Esposito, 1986) and ELISA inhibition dot blot with polyclonal (ELISA-I-DB-p) or monoclonal (ELISA-I-DB-m) antibodies.

3.8 ELISA dipstick sandwich (ELISA-DS) using plastic-bonded nitrocellulose filter paper spotted with polyvalent antibody to human IgG as capture layer and the same antibody labelled with alkaline phosphatase as detecting conjugate; undiluted eluates only were tested and the reactions read by naked eye.

4. RESULTS

The results received at the WHO IRTC were analysed and evaluated with regard to the following criteria:

(a) relative sensitivity of the tests to detect human blood meals;

(b) capacity to distinguish between human and non-human blood sources; and

(c) ability to detect human blood meals in mosquitos at various times after feeding.

The findings of individual investigators are summarized in Table 1.

The relative sensitivity of the tests for detection of human blood, based on the detection of Ig and calculated from blood sample Code No. 8, varied from 1.5 ng to 25 µg/ml of blood (Table 1). The greatest sensitivity was achieved by routine ELISA followed by RPHA and other tests. In three cases, it was impossible to calculate the sensitivity level, because the blood samples were not titrated to the end-point. Therefore, the concentrations of Ig/ml of blood at which the tests were performed were recorded (marked as $\leqslant$ in Table 1). In addition, the sensitivity of two tests was calculated from dilutions of positive human serum controls provided by the laboratories in question; these values, shown in brackets in Table 1, are within the range specified above.

The discrimination between human and non-human blood samples using the tests for detection of human Ig was quite good in all tests. Both human blood samples (Code Nos. 8 and 12) were identified by strongly positive reactions in all the tests. The human component in the mixture of man:cow 1:10 blood sample (Code No. 10) was also positive with all test methods used, while in the 1:100 mixture (Code No. 14) it was identified by weak positive reactions in several but not all tests. There was virtually no cross-reactivity of anti-human Ig antibodies with blood samples from cow, pig, goat and chicken. The only exception was dog blood which cross-reacted by weak reactions in 3 tests as shown in Table 1.

The ability of the tests to detect human blood meals in mosquitos at various times after feeding was very good (Table 1). All the tests showed positive reactions in mosquito specimens killed up to 24 h and all except one test at 32 h after feeding. In a few tests the blood meals were detected at a borderline positivity as late as 48 h after feeding. With one exception, all of the control samples of mosquitos fed on glucose only were negative.

Although the participants were asked to distinguish between human and non-human blood sources, five groups attempted to also identify the non-human blood samples using polyvalent antisera to different animal species. Their findings are summarized in Table 2. A cross-reactivity of antisera to human Ig with dog blood samples was already mentioned above. The cross-reactivity between the blood of sheep and goat is a well-known phenomenon and examples of other cross-reactivities were also identified. However, these cross-reactions were recorded as weak positive reactions and could be corrected by adjusting the cut-off levels of the end-points for positivity.

5. EVALUATION OF TESTS

The performance of the tests carried out on coded samples can be characterized as follows:

The double gel diffusion (DGD) test provided the best results: blood meals were detected in mosquito samples up to 48 h after feeding as well as human components in both mixtures. In addition, it allowed a reliable identification of blood samples of different species. The test is simple to perform without requirements for complicated laboratory equipment and/or electricity and can be completed within 24 h.

The reverse passive haemagglutination assay (RPHA) showed identical results in human/non-human discrimination but had a slight cross-reactivity in identification of other species. The test does not require complicated laboratory equipment and/or electricity, and the results can be read the same day. However, it is not as simple to perform as DGD. It requires sheep red blood cells coated with antibodies (these can be prepared in advance).

The complement fixation test (CFT) provided good results, with a lower sensitivity for the mosquito samples (Code No. 5 - negative). The test is rather complicated since it requires supplies of sheep red blood cells and guinea-pig complement, centrifuge, and a refrigerator. However, it also can be accomplished in 24 h.

The enzyme-linked immunosorbent assays (ELISAs): the direct modification of ELISA was less sensitive than DGD and RPHA to detect the blood in mosquito samples (Code No. 5 - negative) as well as the human component in the man:cow 1:100 mixture (Code No. 14 - negative). This is most likely due to the competition of the species' blood in the mixtures for coating the wells and can be avoided by the inhibition and sandwich modifications of ELISA (ELISA-I and ELISA-S), in which the capture antibodies select and concentrate the antigens present in eluted samples. The ELISAs are relatively simple to perform within 4-6 h and results could be read by eye. The use of instruments (automatic washers, readers, etc.) facilitates the performance and allows more quantitative reading of the end-points. Some of these instruments are available for field use (e.g., battery-operated readers).

The ELISA dot blot (ELISA-DB) and ELISA dipstick (ELISA-DS) modifications are the simplest tests which can be performed within a few hours and read by eye. The nitrocellulose matrix for sandwich assays can be coated with antibodies in advance and stored, rendering the tests highly attractive for use in the field and for the preparation of test kits. The sensitivity of these tests in this trial was quite good and the specificity will depend on the quality of antibodies employed.

Antisera: the key factor for all immunological techniques is always the specificity of antibodies used. Polyvalent antisera to whole serum proteins were originally used for detection of blood meals in precipitin ring tests (Weitz, 1956). Since then, antisera to serum albumin (Kamiyama et al., 1978) or immunoglobulins (Lindquist et al., 1982) were applied. A recent study (Catty, personal communication) revealed that immunoglobulins were better target antigens for the detection of blood meals in mosquitos than albumin, β-lipoprotein, transferrin, haptoglobins and other serum components for the following reasons: immunoglobulins could be detected up to 40 h after feeding, they are sufficiently species-diverse, they are present in high enough concentration in blood meals, and the reagents for their detection are already available. Polyvalent antibodies raised in experimental animals have always to be checked very carefully for specificity and absorbed with cross-reacting antigens by solid phase absorption before use. The use of monoclonal antibodies would improve the specificity, reproducibility and standardization of all the tests because they react specifically with a given epitope of the antigen, they are well defined and can be produced from hybridoma clones at any time. However, the findings of this trial have confirmed a possible cross-reactivity of some monoclonal antibodies, raised against human IgG, with IgG of other species (Jefferis et al., 1982). Therefore, even monoclonal antibodies have to be selected carefully.

Digestion of blood meals: the time interval after feeding at which target antigens could be detected by immunological methods in arthropod vectors will depend on the size of the blood meal and on the rate of the breakdown of antigens. The digestive processes of haematophagous insects have been studied by several investigators (for references see Gooding, 1972). The results of tests carried out in this trial are in accord with the findings published by other investigators (reviewed by Service et al., 1986).

Economically, the total cost of the tests is difficult to calculate because labour costs vary widely from country to country. The cost of consumable materials (e.g., antisera, conjugates, other reagents and materials), calculated for DGD (India), RPHA (Thailand) and ELISA-DB-p (India), did not exceed US$ 0.01 (one cent) per mosquito sample when tests for human/non-human discrimination using polyvalent antisera were employed. The cost of other tests has not yet been calculated. However, higher costs are expected with the introduction of monoclonal antibodies as well as kits ready for use. It has been assumed that the cost would turn out to be in the range of US$ 0.10-0.70 per sample.

6. CONCLUSIONS AND RECOMMENDATIONS

The interlaboratory trial described in this report is a first coordinated attempt to assess the application of immunological techniques for the detection of host blood meals in arthropod vectors at an international level. The main objectives were directed to the detection of host blood meals in mosquitos at various time intervals after ingestion and to the discrimination between human/non-human sources of blood. A total of 14 tests, performed on coded samples by 10 laboratories participating in the trial, have provided the following findings:

(a) human blood meals in mosquitos were reliably detected by all tests up to 24 h after feeding and by all except one test up to 32 h after feeding;

(b) all of the tests discriminated quite well between human and non-human sources of blood (i.e., cow, pig, goat and chicken) with an exception of dog blood samples which reacted slightly with antibodies to human immunoglobulins in 3 tests;

(c) attempts of 5 participating laboratories to identify non-human blood sources (not requested in this trial) were quite successful.

The overall results of the trial have confirmed that even the simplest immunological tests, which do not require complicated laboratory instruments and/or electricity, could be utilized for the reliable detection and discrimination of host blood meals in mosquitos and hopefully in other arthropod vectors. Therefore, the development of simple test kits for selected assay(s), which would facilitate the performance of the tests in endemic areas (i.e., at regional, national and individual levels) appears to be the next logical step.

To achieve this goal, several recommendations have been proposed for future development.

(a) to select in collaboration with appropriate experts suitable test(s) for this purpose;

(b) to prepare and distribute prototype test kits for testing in field trials in endemic areas;

(c) to establish the quality control criteria for the kits in conjunction with reference centre(s) to be created; and

(d) if successful, to initiate regular production of such kits.

Although this project was initially centered around the discrimination of human/non-human blood meals in mosquitos, the identification of non-human blood meals in other vectors could be included, if desired.

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TABLE 1. DISCRIMINATION BETWEEN HUMAN/NON-HUMAN BLOOD SOURCES

Investigator	Tests: Abbreviations[a]	Sensitivity[b] µg Ig/ml	Mosquito samples[c]: M/0	M/8	M/24	M/32	M/48	M/G	Blood samples[c]: Cow	Man	Dog	Mixture Man:Cow (1:10)	Pig	Man	Goat	Mixture Man:Cow (1:100)	Chicken
			1[d]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Catty (& Bray)	ELISA-I-p	0.25	++[e]	++	+	+	0	0	0	++	±	±	0	++	0	±	0
	ELISA-I-m	0.25	++	++	+	±	0	0	0	++	0	±	0	++	0	0	0
	ELISA-I-DB-p	25.0	++	++	+	+	0	0	0	++	0	±	0	++	0	±	0
	ELISA-I-DB-m	25.0	++	++	+	+	0	0	0	++	0	±	0	++	0	0	0
Collins	DGD-p	2.0	++	++	++	+	±	0	0	++	0	+	0	++	0	±	0
Esposito	ELISA-DB-PAP-p	0.75	++	++	+	++	0	0	0	++	0	++	0	++	0	±	0
Lambert	ELISA-p	0.0015	++	++	+	±	0	0	0	++	0	+	0	++	0	0	0
	ELISA-DB-p	⩽15.0 (0.15)	++	++	+	+	±	0	0	++	0	+	0	++	0	0	0
Petchclai	RPHA-p	0.075	++	++	+	+	±	0	0	++	0	+	0	++	0	±	0
Roberts	ELISA-DS-p	⩽375.0 (<20.0)	++	++	+	+	±	0	0	++	0	++	0	++	0	0	0
Service	ELISA-S-m	0.375	++	++	±	0	0	0	0	++	±	++	0	++	0	+	0
Sharma	ELISA-DB-p	0.375	++	++	++	+	0	+	0	++	0	+	0	++	0	0	0
Staak	CFT-p	7.5	++	++	+	+	0	0	0	++	0	±	0	++	0	±	0
	ELISA-p	⩽57.0	++	++	±	±	0	0	0	++	±	±	0	++	0	0	0

LEGEND TO TABLE 1

[a] Abbreviations for tests:

CFT = Complement fixation test
DGD = Double gel diffusion
ELISA = Enzyme-linked immunosorbent assay
ELISA-DB = ELISA dot blot
ELISA-DB-PAP = ELISA dot blot peroxidase-anti-peroxidase
ELISA-DS = ELISA dipstick sandwich
ELISA-I = ELISA inhibition
ELISA-I-DB = ELISA inhibition dot blot
ELISA-S = ELISA sandwich
RPHA = Reverse passive haemagglutination assay
p = polyclonal antibodies used
m = monoclonal antibodies used.

[b] Relative sensitivity of tests was calculated under the assumption that human blood sample Code No. 8, obtained by punch in the middle of the spot, contained 10 µl of blood and that 1 ml of human blood contained 7.5 mg of immunoglobulins (Ig). The sensitivity represents the lowest concentration of Ig (µg per 1 ml of blood) detectable by the test or the concentration at which the test was performed (marked as ≤ in the table); the values, shown in brackets, were calculated from dilutions of human serum samples provided by the laboratories.

[c] Mosquito samples: M/0 to M/48 = individual mosquitos, membrane-fed on human blood and killed at various time intervals after feeding (e.g., 0, 8, 24, 32 and 48 hours); M/G = mosquitos fed on glucose only and killed immediately. Blood samples = samples of blood (or mixtures) from different species as indicated.

[d] Code numbers for specimens (i.e., 1-15).

[e] Intensities of reactions are expressed as strongly positive (++), positive (+), weakly positive (±) and negative (0). These correspond to dilutions or to visual intensity of reactions in dot blot tests and dipstick tests.

TABLE 2. IDENTIFICATION OF SPECIES

Tests[a]	Blood samples[b]								
	Cow 7[c]	Man 8	Dog 9	Mixture Man:Cow (1:10) 10	Pig 11	Man 12	Goat 13	Mixture Man:Cow (1:100) 14	Chicken 15
ELISA-I-p	Cow[d]	Man (Chicken)	Dog (Man)	Cow Man	Pig	Man (Chicken)	Sheep	Cow Man	Chicken
DGD	Cow	Man	Dog	Cow Man	Pig	Man	Goat	Cow Man	Avian
ELISA-DB-PAP	Cow	Man	Dog	Man	Pig	Man	Sheep (Dog)	Man	Not identified[e]
RPHA	Cow	Man (Cow)	(Man)[f]	Cow Man	Pig	Man	Goat (Cow)	Cow Man	Cow[e]
CFT	Ruminants[g]	Man	Dog	Ruminants Man	Pig	Man	Ruminants	Ruminants Man	Chicken
ELISA	Cow	Man (Dog)	Dog (Man)	Cow Man	Pig	Man (Dog)	Ruminants	Cow	Bird

Legend:

a Abbreviations for tests described in Table 1.

b Blood samples or mixtures from different species as indicated.

c The numbers 7 to 15 are code numbers identical with text and Table 1.

d Species identified by investigators; cross-reactions recorded in brackets.

e Not tested for chicken or avian.

f Not tested for dog.

g Antisera to ruminants contained antibodies to cow and goat serum proteins.