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CURRENT AND POSSIBLE FUTURE TECHNIQUES FOR THE
IDENTIFICATION OF BLOOD MEALS OF VECTOR HAEMATOPHAGOUS ARTHROPODS

by

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The issuance of document WHO/MAL/83.992 (WHO/VBC/83.873) on the use of the gel diffusion technique for the identification of blood meals by Collins et al. (1983) prompted us to compile a review of the current techniques used by the Bloodmeal Identification Unit at the Imperial College, which was started by Weitz (1952, 1956), continued by Boreham (1972) and, since 1979, was placed under our responsibility.

Our aims have been the following:

- (a) To find the best means of immunizing rabbits with animal sera to ensure serum of a high titre and a relatively high specificity.
- (b) To establish a reliable method for detecting precipitins which requires only very small amounts of antigen.
- (c) To find a technique with sufficient specificity that enables to distinguish between different species of animals (e.g. sheep and goats).

1. PREPARATION OF RABBIT ANTISERA

A rabbit is inoculated intravenously with 1 ml of serum of another animal or 2 ml of a solution of 2.5 g animal serum albumin in 10 ml of phosphate-buffered saline (PBS). A further five intravenous injections of 0.5 ml of serum or 1 ml albumin solution are administered over a period of two and a half weeks.

Two and three weeks after the last inoculation, blood is drawn, serum separated and tested by the precipitin ring test (Weitz, 1956; Boreham, 1972) (see below) to determine the titre of the antiserum. If the titre is 1:8 or 1:16, the rabbit is bled and the serum stored. It has been found that the method usually yields antiserum with cross-reactions to other animal sera considerably lower than the homologous reaction. The best stimulating antigen giving the lowest cross-reactions appears to be albumin which we prepare by salt fractionation. This antiserum is also tested against animal sera related to the specific serum used as antigen. The serum is used at a dilution at which the homologous reaction is strong but at which cross-reactions do not occur. If any serum other than the specific serum reacts with the antiserum at the same titre as the specific serum, the antiserum may be absorbed by adding a calculated amount of cross-reacting animal serum to a known volume of antiserum (see Weitz, 1952). The mixture is allowed to react at 4°C in the refrigerator overnight and then is spun at 12 000 g for 30 min at 4°C and the supernatant aspirated and tested again to determine if the cross-reactivity of the antiserum has been absorbed. However, this method is avoided if possible.

The antiserum may be freeze-dried or, more conveniently, stored in aliquots of 4 ml each at -40°C or below. It is used diluted, the most common dilution being 1:8.

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2. PRECIPITIN RING TEST AND TESTING PROCEDURE

The stomach contents of the haematophagous arthropods to be tested are smeared on to circular Whatman No. 1 filter papers (9-10 cm in diameter) about 0.5-1 cm from the edge of the paper, and approximately 2 cm apart (smears of 16 mosquitos or eight tsetse flies or triatomine bugs can be accommodated on each filter paper); these are then dried over silica gel in a cool place and sent to the Unit for identification.

For small insects such as Culicoides, Simulium and phlebotomine sandflies, the blood meals are not smeared on filter papers. Instead, two grains of dried silica gel are put into a gelatin capsule and covered with a small piece of non-absorbent cotton-wool on which either the whole insect or its abdomen is placed; the capsule is then sealed and labelled.

The blood-meal smear or whole insect is eluted in saline (0.5 ml for a mosquito smear and 1 ml for a smear of a tsetse fly or a triatomine bug) and the elution is left overnight at 4°C in a refrigerator.

The liquid precipitin ring test, commonly known as the precipitin ring test (Weitz, 1952, 1956; Boreham, 1972) is used in identifying larger blood meals. It consists of drawing 50 µl of saline extract of the blood meal into a capillary pipette, followed by 50 µl of a selected dilution of the antiserum. If the test system is homologous, i.e. if the blood meal is derived from a particular host for which the antiserum was prepared, a white band of precipitate forms at the interface between the blood meal extract and the antiserum (at room temperature, after 15-30 min with a highly concentrated blood meal and after 2 h with a lower quantity of immunoreactant in the blood meal). This test is sensitive and easy to perform with the automatic dispensing machine invented by Weitz (1957). Antiserum dilutions which react with serum at 1:50 are used to measure potency of the antiserum and the method of Weitz (1952, 1956, 1957) and Boreham (1972), using dilution of the antigen, is no longer used.

After 10-15 tests, the blood-meal extract of even an insect as large as a tsetse fly is exhausted and a technique which requires much less antigenic material was sought with the valuable assistance of the Forensic Laboratory of the Metropolitan Police, London. After considerable experimentation with gels and buffers, the counter-current immunoelectrophoresis (CCIE) method of precipitation in gel appeared to be the method of choice (see description below).

3. COUNTER-CURRENT IMMUNOELECTROPHORESIS (CCIE)

The method is based on the principle that the albumin of the blood meal (antigen) in the cathodic well migrates towards the anode by electrical attraction and the immunoglobulin (antibody) in the anodic well is drawn towards the cathode by electro-osmotic flow through a supporting medium (e.g. a gel of agar and agarose in the proportion of 2:1 in a sodium barbiturate buffer at pH 8.6). The antigen and antibody meet between the opposing wells in a relatively short time, causing a precipitin band to form (Culliford, 1964).

3.1 Method and procedure

A mixture of 0.67 g of pure agar (Oxoid, code L28) and 0.33 g of agarose (BDH, Poole) is boiled in 50 ml distilled water mixed with 50 ml gel buffer. The molten gel is degassed at reduced pressure to remove air bubbles. Electrophoretic plates (75 mm x 50 mm) are cleaned, polished dry with methylated spirit and 7 ml of the hot molten gel is poured on to each plate and spread to an even thickness of 1 mm; the plates are numbered on the upper right-hand corner. When the gel has set, the plates are transferred to a humid box for half an hour.

Wells, 1.5 mm in diameter and 2 mm apart, are punched in the gel and filled with blood-meal extract (antigen) and antiserum; the plates are then inverted and placed on the bridge of an electrophoretic tank so that numbers on the upper right-hand corner of each plate appear on the bottom right-hand corner. Three-quarters of the electrophoretic tank is filled with a sodium barbiturate buffer of pH 8.6 (see Appendix). An electric current of 20 mA at 100 volts is passed for 15 min through four plates simultaneously. The plates are then washed twice at two-hour intervals with saline and finally with distilled water. The

gel surfaces of the plates are covered with filter paper dampened with distilled water and left overnight to dry. The dried plates are then stained with Amido black or Coomassie brilliant blue (see Appendix), a procedure which takes 10-15 min. The excess stain is washed off with de-staining solution (see Appendix). The stained plates are then examined for precipitation through a viewer or against a white background.

This technique has the advantage of requiring only 5 µl of blood-meal extract and is a means by which mixed meals (i.e., obtained by feeding on more than one host) can be readily demonstrated (Dhanda & Gill, 1982; Guy et al., in press). It has been found to be as sensitive as the precipitin ring test but, of course, confers no greater specificity and the problem of distinguishing between some African ungulates, sheep and goats, ox and buffalo and members of the Suidae remains. Increased specificity can be obtained by means of the haemagglutination test.

4. PASSIVE HAEMAGGLUTINATION INHIBITION TEST (PHI)

Sheep erythrocytes are coated, by tanning, with serum of the host species being tested. They can be agglutinated by the corresponding antiserum. The agglutination can be inhibited or not by a selected concentration of antiserum reacted with varying dilutions of homologous serum. At the optimum dilution of antiserum, i.e. when the antiserum/antigen mixture still produces a positive reaction to antigen-coated erythrocytes, the inhibition of agglutination of sensitized erythrocytes by the test antigen is species specific. By means of this species-specific inhibition of agglutination by blood-meal extract the host can be identified (Weitz & Jackson, 1955; Weitz, 1956).

5. FUTURE DEVELOPMENTS

Even with the haemagglutination inhibition test, which is both time consuming and difficult to perform, not all the problems of differentiation are solved.

Further differentiation seems possible using either monoclonal antibodies against albumins of the relevant animal sera, or iso-electrofocusing haemoglobin-containing gel and/or serum iso-enzymes to obtain a pattern by which the species of the donor host can be identified. Albumins are selected as stimulants of possible monoclonal antibodies since albumin is the last substance digested in the gut of arthropods (Williams, 1956) and reacts with the antibody in the CCIE test. In the case of the monoclonal antibody test, one would have to presuppose that of the 10-20 antigenic determinants of the albumin of, for example, sheep at least one does not exist in the goat and which could elicit a B cell proliferation response in a BALB/c mouse. Work to produce monoclonal antibodies to the albumins of selected animal sera has now commenced.

Electrophoretic patterns of haemoglobin or iso-enzymes will then be explored to determine whether it might be possible to identify a pattern specific to a single species of animal and even to the blood of selected individual donors of the same species.

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APPENDIX

<u>Buffer formulae</u>	<u>Tank pH 8.6</u>	<u>Gel pH 8.6</u>
Sodium diethylbarbiturate	17.52 g	14.0 g
Diethylbarbituric acid	2.76 g	2.2 g
Calcium lactate	0.77 g	2.0 g
Distilled water	2 litres	2 litres (double strength)

Protein stains

(i) Amido black

Naphthaline black 0.2 g

Methyl alcohol 100 ml

Glacial acetic acid 20 ml

Distilled water 100 ml

(ii) Coomassie brilliant blue

Coomassie blue (R250 for electrophoresis) = 0.63 g

10% acetic acid = 25 ml

25% isopropyl alcohol = 63 ml

Make up to 250 ml with distilled water; filter and store

De-staining solution:

Methyl alcohol 500 ml

Glacial acetic acid 100 ml

Distilled water 500 ml

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