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ROGER M. MCKINNEY ET AL

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9. ABSTRACT

A procedure was developed for specific fluorescent antibody staining of erythrocytes from various mammalian species for the purpose of identifying the source of mosquito blood meals. Antibodies were prepared in rabbits against the erythrocyte membranes of humans, cows, horses, pigs, and sheep. The serum globulin fractions were labeled either with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate, each of which produced brilliant fluorescent antibody staining of homologous erythrocytes. Although initially crossreactive, the conjugates were rendered specifically reactive, among the species tested, by adsorption with stroma preparations of erythrocytes from the heterologous species. A blood meal extracted from a single mosquito, when diluted with saline, was sufficient to make eight microscope slide preparations. By pairing and mixing fluorescein labeled antibody to erythrocytes of one species with rhodamine labeled antibody to erythrocytes of another species, it was possible to test for two species with a single microscope slide preparation, thus allowing one to screen for 16 animal species with a blood meal extracted from a single mosquito. Blood meals were identified by this technique up to 44 hours after ingestion by the mosquito.

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Technical Development Laboratories  
Malaria Program  
Center for Disease Control  
Box 2167 • Savannah, Ga. 31402

ROGER M. MCKINNEY, JANET T. SPILLANE, AND PRESTON HOLDEN

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## MOSQUITO BLOOD MEALS: IDENTIFICATION BY A FLUORESCENT ANTIBODY METHOD\*

ROGER M. MCKINNEY,<sup>†</sup> JANET T. SPILLANE, AND PRESTON HOLDEN<sup>‡</sup>  
*Technical Development Laboratories, Malaria Program, Center for Disease Control,  
Savannah, Georgia 31402, and Arboviral Disease Section, Ecological Investigations  
Program, Center for Disease Control, Fort Collins, Colorado 80521*

**Abstract.** A procedure was developed for specific fluorescent antibody staining of erythrocytes from various mammalian species for the purpose of identifying the source of mosquito blood meals. Antibodies were prepared in rabbits against the erythrocyte membranes of humans, cows, horses, pigs, and sheep. The serum globulin fractions were labeled either with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate, each of which produced brilliant fluorescent antibody staining of homologous erythrocytes. Although initially cross-reactive, the conjugates were rendered specifically reactive, among the species tested, by absorption with stroma preparations of erythrocytes from the heterologous species. A blood meal extracted from a single mosquito, when diluted with saline, was sufficient to make eight microscope slide preparations. By pairing and mixing fluorescein labeled antibody to erythrocytes of one species with rhodamine labeled antibody to erythrocytes of another species, it was possible to test for two species with a single microscope slide preparation, thus allowing one to screen for 16 animal species with a blood meal extracted from a single mosquito. Blood meals were identified by this technique up to 44 hours after ingestion by the mosquito.

A means of identifying insect blood meals is a valuable tool in studying the role of insects and their hosts as vectors and reservoirs of disease. The most widely used method of identifying insect blood meals has been the precipitin test. The method, as discussed in detail by Weitz,<sup>1</sup> involves removing the abdomen of the blood-fed insect and squeezing the contents onto filter paper. The filter paper is later extracted with saline. Portions of the saline extract are used in the precipitin test with antisera prepared in rabbits against the sera of the various species suspected of being hosts to the mosquito. Tempelis and Lofy report improved specificity by using antisera prepared in chickens as opposed to rabbits.<sup>2</sup> However, they observed considerable cross-reactivity among mammalian species belonging to the same families and were not able to distinguish between blood meals of sheep and cattle. More recently, Tempelis and Rodrick described a passive hemagglutination inhibition technique with which they were

able to differentiate feedings on different hosts that could not be separated by the precipitin test.<sup>3</sup> A fluorescent antibody (FA) method has been described for identification of blood meals, that involves staining of the soluble antigen or serum of the blood meal.<sup>4</sup> Miki et al.<sup>5</sup> investigated the use of fluorescent antibodies to identify the origin of blood stains for possible application in the field of legal medicine. Although they obtained specific staining of blood crusts by direct FA staining, they were able to stain red blood cells which had been washed free of serum only by an indirect method using unlabeled rabbit anti-serum against the blood cells and fluorescent anti-rabbit serum. There have been a number of reports concerning fluorescent antibody staining of red blood cells for the purpose of identifying or demonstrating the presence of the various erythrocyte antigens of human red cells, but with no application to species identification.<sup>6-9</sup> We now report a direct FA method for identification of mosquito blood meals based on the use of erythrocyte membranes of the host species as the antigen.

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\* Please address requests for reprints to: Miss Janet T. Spillane, Technical Development Laboratories, Malaria Program, Center for Disease Control, P. O. Box 2167, Savannah, Georgia 31402.

<sup>†</sup> Present address: Laboratory Division, Center for Disease Control, Atlanta, Georgia 30333.

<sup>‡</sup> Dr. Holden died 17 May 1972.

### MATERIALS AND METHODS

#### *Preparation of Antigens*

Erythrocyte membranes or stroma from cow, horse, man, hog, and sheep were prepared free of leucocytes generally as described by Maddy.<sup>10</sup>

Cow and horse erythrocyte membranes were solubilized by partitioning between butanol and water.<sup>10</sup> The water soluble fraction was lyophilized and used as the antigen. In the case of man, hog, and sheep, lyophilized preparations of whole erythrocyte membranes were used as the antigen. In either case the antigen could be stored for long periods at 5°C with no apparent deterioration.

#### *Preparation of Antisera and Conjugates*

New Zealand albino rabbits were used as antibody producers. The most satisfactory immunization schedule used was a primary intramuscular injection of 25 mg of lyophilized stroma or stroma extract homogenized with 0.5 ml of normal saline and 0.5 ml of Freund's complete adjuvant, followed by two intravenous boosters, at 6-week intervals, of 25 mg of erythrocyte stroma or extract homogenized in 0.7 ml of saline. Seven days after each booster, about 50 ml of blood was taken from each rabbit, the serum was collected, and the globulin fraction obtained by two precipitations with 40% saturated ammonium sulfate. The globulin fractions were labeled with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate. Fluorescein conjugates were prepared so as to give approximately 15  $\mu\text{g}$  of bound fluorescein/mg of protein.<sup>11</sup> The rhodamine conjugates were prepared by adding tetramethylrhodamine isothiocyanate as a dry powder to the globulin in a ratio of 25  $\mu\text{g}$ /mg of protein. The reaction was run in a carbonate buffer at pH 9.5 for 16 hours at room temperature, and insoluble material was removed by centrifugation. Labeled protein was separated from unreacted dye by gel filtration with Sephadex G-50.\* Eluted conjugates contained approximately 0.3% protein.

In general, unabsorbed fluorescein and rhodamine conjugates gave cross-staining among the mammalian species studied. Conjugates were therefore absorbed with lyophilized stroma of other species. For each of the five species against which antisera were prepared, 2 ml of undiluted conjugate were absorbed by a mixture of 10 mg of lyophilized erythrocyte stroma of each of the other four species. The mixtures were maintained

at room temperature for 2 or 3 hours and then stored overnight in the refrigerator at 5°C. The absorbed conjugates were then separated by centrifugation and filtration through a 0.8- $\mu$  Millipore filter and diluted 1:5 with 0.5% bovine serum albumin. A rhodamine conjugate for one species was then combined with a fluorescein conjugate for another species in a 1:1 ratio or a final dilution of 1:10 with respect to each conjugate.

#### *Preparation of Slides*

Samples of whole blood from cow, horse, man, pig, and sheep were diluted with saline for microscope slide preparation. The air-dried blood smears were fixed by immersing in acetone at room temperature for 1 minute. Slides could then be stained directly or stored in the refrigerator at 5°C.

Preparations were also made in which equal parts of blood of two different animal species were mixed in order to test the possibility of identifying two different antigens on a single slide by using the appropriate paired and mixed fluorescein and rhodamine conjugates.

Blood meals were obtained by allowing *Culex quinquefasciatus* mosquitoes to feed on humans, pigs, and cows. No feedings were obtained from horses or sheep. The blood-fed mosquitoes were held at room temperature or approximately 25°C. After various digestion time intervals from 1 to 44 hours, the abdomens were removed and the blood contents pressed into 0.5 ml volumes of saline. In each case the saline mixture was distributed on inscribed circles on four slides, making two smears per slide, or a total of eight smears from each mosquito. After air drying, the preparations were fixed in acetone and stained by conventional FA technique.

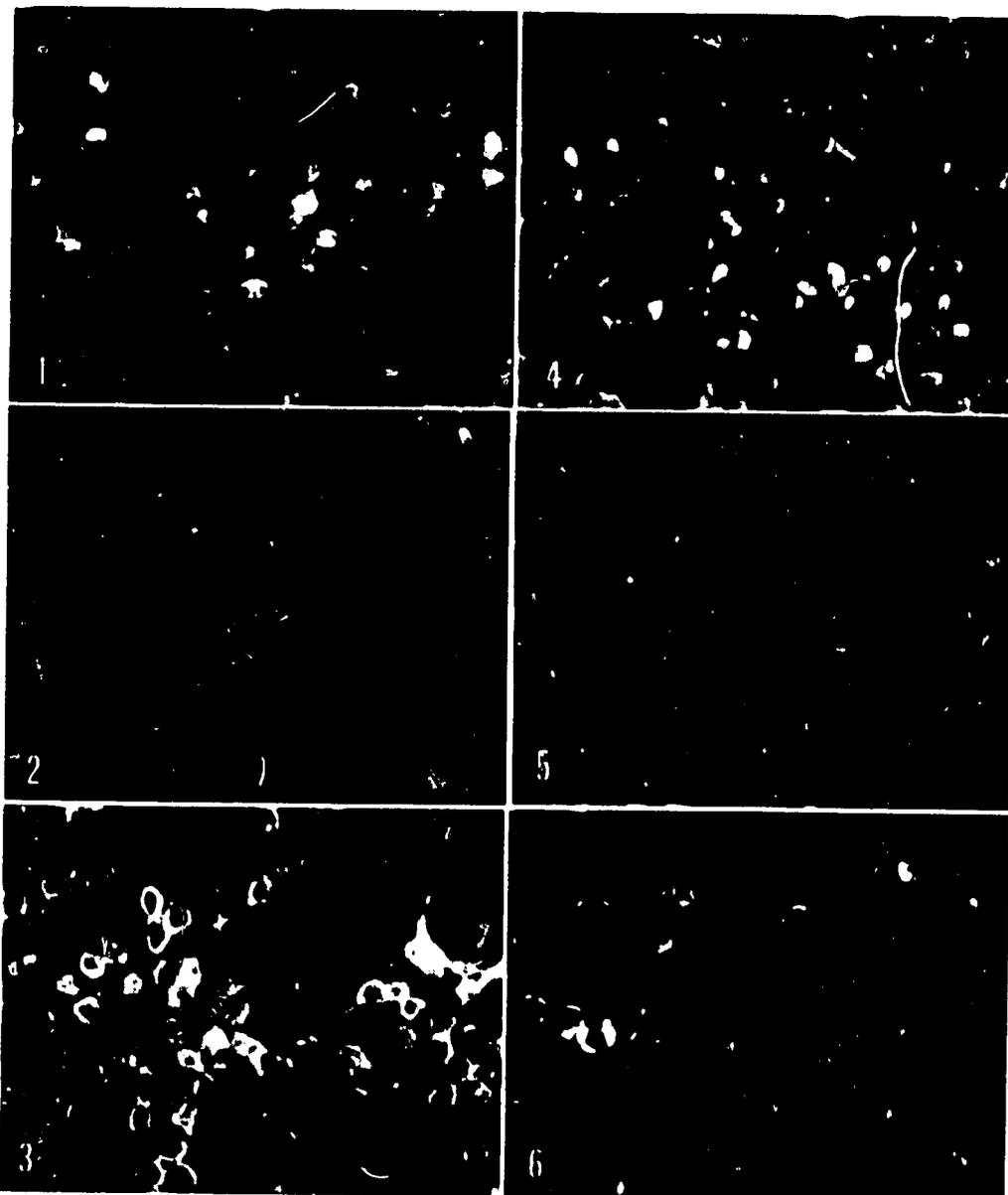
#### *Storage of Mosquito Blood Meals and Slides*

Storage tests were conducted by holding intact blood-fed mosquitoes in screw cap vials at slightly above 0°C (wet ice). Blood meals from individual mosquitoes were extracted and subjected to FA staining at intervals up to 72 hours. Also, acetone-fixed slides of mosquito blood meals were stored at 5°C and tested at 1-week intervals up to 10 weeks for FA staining.

#### *Fluorescence Microscopy*

A Leitz Ortholux microscope with a Ploem-type<sup>12</sup> vertical illuminator was used to observe

\* Use of trade names is for identification purposes only, and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.



FIGURES 1 to 6. 1. Mixed human and bovine erythrocyte membranes. Human cells specifically stained with rhodamine labeled anti-human erythrocyte antibody (red fluorescing) and bovine cells specifically stained with fluorescein labeled anti-bovine erythrocyte antibody (green fluorescing). Two exposures were used on a single frame with excitation at 545 nm for rhodamine and then excitation at 490 nm for fluorescein.  $\times 756$ . 2. Human erythrocyte membranes stained with rhodamine labeled anti-human erythrocyte antibody. Fluorescein-stained bovine red cells are present in the field but are excluded by the filter system.  $\times 756$ . 3. Mixed sheep and bovine erythrocyte membranes. Sheep cells are stained with fluorescein labeled anti-sheep erythrocyte antibody and bovine cells are stained with rhodamine labeled anti-bovine erythrocyte antibody. Excitation with blue light and K 530 barrier filter allows simultaneous observation of both fluorescein and rhodamine fluorescence. Rhodamine fluorescence is dull brick-red with this system.  $\times 933$ . 4. Mixed sheep and bovine erythrocyte membranes. Sheep cells are stained with fluorescein labeled anti-sheep erythrocyte antibody and bovine cells are stained with rhodamine labeled anti-bovine erythrocyte antibody. Two exposures were used on a single frame with excitation at 545 nm and then excitation at 490 nm to give maximum excitation of both fluorescent labels.  $\times 756$ . 5. Bovine blood meal taken from a mosquito after 25 hours digestion time specifically stained with rhodamine labeled rabbit anti-bovine erythrocyte antibody.  $\times 756$ . 6. Human blood meal taken from a mosquito after 44 hours digestion time specifically stained with fluorescein labeled rabbit anti-human erythrocyte antibody.  $\times 756$ .

the slides. By using blue excitation light (2 Leitz KP 490 primary filters, 495 nm interference plate, and K 530 barrier filter), the green fluorescence of fluorescein and the dull red fluorescence of rhodamine could be observed simultaneously. By using green excitation light (2 mm BG 36 + AL 546 primary filters, 580 nm interference plate, and K 590 barrier filter), the bright red fluorescence of rhodamine was observed without interference from fluorescein.

#### RESULTS

Cross-staining of some species was observed with unabsorbed conjugates. Very strong cross-staining was encountered when anti-sheep conjugates were applied to bovine red cells, and moderate cross-staining was observed with anti-bovine conjugates on sheep red cells. Absorption of anti-sheep conjugate with bovine red cell stroma, in one case, completely removed the cross-staining of bovine red cells, and retained bright specific staining for sheep red cells. In a second case, absorption of an anti-sheep conjugate with bovine red cell stroma completely removed the staining capacity for bovine red cells but left only a very weak staining capacity for sheep red cells, demonstrating individual difference in the response of rabbits to the red cell antigen. All other crosses, including the cross-staining of sheep red cells by anti-bovine conjugates, were readily removed by absorption with the lyophilized red cell stroma of heterologous species, while bright specific staining was retained. Figure 1 is a photograph of a mixture of human and bovine red cells simultaneously stained by a mixture of rhodamine anti-human conjugate and fluorescein anti-bovine conjugate. The larger red fluorescing cells are the human erythrocytes. The photograph was obtained by a double exposure first using the green excitation light, then changing the filters to use the blue excitation light. Figure 2 is a photograph of another field of the same slide, obtained by using just the green excitation light and K 590 barrier filter. The bovine red cells are completely excluded by this filter system, demonstrating that no cross-staining of bovine red cells with the rhodamine labeled anti-human conjugate is evident. Figures 3 and 4 are photographs of a mixture of sheep and bovine red cells simultaneously stained by a mixture of rhodamine anti-bovine conjugate and fluorescein anti-sheep conjugate. Figure 3 was obtained by using blue

excitation light for maximum excitation of fluorescein, and a K 530 barrier filter which allows observation of both fluorescein and rhodamine fluorescence. The bovine red cells, in this case, appear dull brick-red since the blue light does not produce maximum excitation of rhodamine. Figure 4 was obtained by double exposure, in the same manner as was used for Figure 1, in order to show the rhodamine labeled bovine cells and fluorescein labeled sheep cells with maximum fluorescence on a single photograph.

Bright specific staining was obtained for mosquito blood meals in all cases up to 24 hours of digestion time. In cases where the mosquito was well engorged initially, good staining was observed after 44 hours of digestion time. Where digestion progressed to partial fragmentation of the erythrocytes there were usually sufficient intact cells remaining for easy identification. In Figure 5 we have shown a bovine blood meal stained with a rhodamine anti-bovine conjugate after 24 hours digestion time. Figure 6 is a human blood meal stained with a fluorescein anti-human conjugate after 44 hours digestion time.

Blood meals obtained from intact mosquitoes stored at wet-ice temperature for 72 hours gave bright FA staining. Acetone-fixed slides of mosquito blood meals stored for 10 weeks at 5°C showed no loss in brightness of FA staining.

#### DISCUSSION

Although only five mammalian species have been studied to date, it appears that conjugates of good specificity can be prepared by using the erythrocyte membrane as the antigen. The easy distinction of bovine erythrocytes from those of sheep by the use of absorbed conjugates demonstrates the high specificity obtainable by the FA method.

By using eight blood meal smears from each mosquito and pairs of mixed rhodamine and fluorescein labeled conjugates as described, one mosquito blood meal can be used to screen for 16 mammalian species. It is also possible to identify mixed blood meals where a mosquito has fed successively on two different host species.

We emphasize the necessity of using acetone fixation to obtain bright staining. This requirement suggests that the antigen responsible for FA staining is not a surface antigen, but is exposed upon reaction of the erythrocyte membrane surface with acetone.

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