

# Host Antibodies in Mosquito Bloodmeals: A Potential Tool to Detect and Monitor Infectious Diseases in Wildlife

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**ABSTRACT** When a female mosquito bites, it carries away a blood sample containing specific antibodies that can provide a history of the immune responses of its vertebrate host. This research examines the limits and reliability of a technique to detect antibodies in blood-fed mosquitoes in the laboratory. Mosquitoes were fed on blood containing a specific antibody, and then they were assayed using an enzyme-linked immunosorbent assay to determine the limits of detection of antibody over time, at different temperatures and initial antibody concentrations. The antibody, at an initial concentration of 1  $\mu\text{g/ml}$ , could be detected in mosquitoes for 24–48 h after feeding. Blind tests simulating the assay of feral mosquitoes were used to test the reliability of the method and detected positive mosquitoes with few false negatives and no false positives. Specific antibodies also could be detected in mosquitoes that had been air-dried or preserved in ethanol. This research indicates that, in theory, the collection and immunological assay of blood-fed mosquitoes could be developed to detect and monitor infectious disease in wildlife.

**KEY WORDS** mosquito, enzyme-linked immunosorbent assay, bloodmeal, antibody, wildlife disease

Human health and livestock production can be affected by infectious diseases of wildlife living close to human habitations and livestock operations. Expanding development, climate change, the translocation of animals, and the adaptation of wildlife to urbanized environments have increased the exposure of humans to zoonotic diseases such as hantavirus, Lyme disease, avian influenza, and rabies. Many livestock diseases may originate in neighboring wildlife, including bovine tuberculosis, brucellosis, anaplasmosis, Newcastle disease, and prion diseases. Infectious diseases are also a concern in the conservation of wildlife already threatened by habitat loss and exploitation. Significant population declines in many species, including great apes (Wolfe et al. 2001, 2002; Kilbourn et al. 2003) and sea lions (Burek et al. 2003) have been associated with disease. The ability to detect and monitor such diseases in wildlife species is essential to reduce transmission to humans and livestock and within wildlife populations.

Surveillance of wildlife disease commonly involves trapping or killing large numbers of animals for direct sampling of blood and tissues. This can be difficult, expensive, and dangerous to field personnel who handle animals and can expose them to the risk of zoonotic disease. Sampling of wildlife may be unacceptable in parks and wildlife reserves, and capture and invasive sampling methods may be inappropriate for endangered species.

Blood-fed mosquitoes captured in the wild contain a sample of blood taken from vertebrate hosts. This blood contains specific immunoglobulins (Igs) which, if detectable, can provide a history of the immune responses of the vertebrate host. If antibodies, specific to particular disease agents, can be reliably detected in blood-feeding arthropods then the collection and immunological assay of mosquitoes or other hemophagous arthropods could be used to detect and monitor pathogens in wildlife. This would provide an alternative to capturing or killing to monitor the health of wildlife.

Several avenues of research including the use of host antibodies to control vector mosquitoes have demonstrated that specific host antibodies persist and can be detected in arthropod bloodmeals, in the hemolymph, and bound to gut epithelia (Nogge and Giannetti 1980, Ackerman et al. 1981, Minoura et al. 1985, Hatfield 1988, Tesh et al. 1988, Lackie and Gavin 1989, Vaughan et al. 1990).

Fujisaki et al. (1984) detected specific antibodies to the pathogenic piroplasm *Theileria sergenti* in the hemolymph of ticks that had fed on experimentally infected calves, and Vaughan and Azad (1988) detected an antibody to *Rickettsia typhi*, the agent of murine typhus, in bloodmeals of five species of mosquitoes that had fed on experimentally infected rats. Tesh et al. (1988) examined the disappearance of albumen, IgG, and IgM after mosquitoes and sand flies fed. They found that unlike ingested albumen, IgG and IgM remained at detectable levels for several days. Hatfield

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(1988) identified specific antibodies in the hemolymph of *Aedes aegypti* (L.) up to 48 h after feeding. Lackie and Gavin (1989) reported persistence of specific host antibodies in whole-body homogenates of *Anopheles stephensi* Liston up to 9 d after feeding, and they found that the antibody was present in the hemolymph after it had disappeared from the gut. Vaughan et al. (1990) studied the movement of rat antibodies specific to *Plasmodium falciparum* into the hemolymph of *An. stephensi* finding that, at peak titers, the hemolymph contained  $\approx 0.5\%$  of the antibody concentration found in the host serum. In all these studies, normal, pathogen-specific antibody concentrations found in blood were detectable in the arthropods 24–48 h after the bloodmeal was taken.

These studies used various serological techniques, including immunoprecipitation (Fujisaki et al. 1984, Minoura et al. 1985) immunofluorescent antibody technique (Schlein et al. 1976, Ackerman et al. 1981, Fujisaki et al. 1984, Vaughan and Azad 1988), radioimmunoassay (Ben-Yakir; 1989), and enzyme-linked immunosorbent assay (ELISA) (Ben-Yakir et al. 1987, Hatfield 1988, Lackie and Gavin 1989, Vaughan et al. 1998). ELISA is considered to be the most specific, sensitive, and useful in the field (Burkot et al. 1981, Service et al. 1986, Beier et al. 1988).

These studies of host antibodies in the bloodmeal and tissues support the concept of using host antibodies in arthropods to detect infectious disease. Our study explores the feasibility of adapting existing techniques for this purpose, using a simple ELISA that is straightforward, economical, and can be modified for use in the field. This ELISA also can handle large numbers of samples. We determined the limits to detection of a specific antibody in mosquito bloodmeals by using established digestion curves similar to those used in many of the above-mentioned studies, and those limits were then used in blind tests to simulate testing wild caught mosquitoes to provide a proof-of-concept.

### Materials and Methods

**Mosquitoes and Rearing.** *Aedes aegypti* (L.) (black-eyed Liverpool strain) were maintained throughout their life cycle in an incubator at 27°C, 60–70% RH, and a photoperiod of 12:12 (L:D) h. Larvae were reared on ground TetraMin fish food, and the adults were maintained on 10% sucrose solution provided in cotton balls.

**Blood Feeding.** Adult mosquitoes, 2–4 d old, were starved for 24 h and then they were fed on an artificial feeder that held blood at 37°C. Parafilm was stretched across the mouth of the tube, and blood was placed onto the upper surface of the membrane. Mosquitoes held in paper cups with a mesh top fed through this membrane from below.

Mosquitoes were allowed to feed for 30 min on citrated human blood from one of us (B.J.L.) to which a mouse IgG1 monoclonal anti-chicken egg albumin antibody (mAb) (A6075, Sigma-Aldrich, St. Louis, MO) had been added. This mAb was diluted from an

initial concentration of 10 mg/ml to a 1:10 dilution in phosphate-buffered saline with Tween 20 (PBS-T) in working aliquots and stored frozen. For feeding, the mAb was diluted in blood to a final dilution of 1:1,000 (10  $\mu\text{g/ml}$ ) or 1:10,000 (1  $\mu\text{g/ml}$ ).

Blood-fed mosquitoes were maintained as described above for specified times, and then they were frozen and stored at  $-80^\circ\text{C}$  in 1.5-ml tubes. Subsequently, each mosquito was homogenized with a tissue grinder in a 1.5-ml tube in 200  $\mu\text{l}$  of PBS-T, centrifuged at  $16,000 \times g$  for 3 min at  $4^\circ\text{C}$ , and the supernatant was used directly in the ELISA.

**Controls.** A sample of blood containing the antibody was retained for use as a positive control in each ELISA. This sample was held in a  $37^\circ\text{C}$  water bath while the mosquitoes were fed. Negative controls included blood without the antibody, an isotype mouse IgG1 control (M5284, Sigma-Aldrich) at a 1:1000 dilution in PBS-T, and a PBS-T blank was used to zero the plate reader. All controls were frozen at  $-80^\circ\text{C}$ . The isotype mouse IgG was used to determine the background levels for the assay, and the positive and negative control blood samples were diluted to 200  $\mu\text{l}$  in PBS-T before the assay. The volume of blood was based on the estimated, average bloodmeal volume from a sample of mosquitoes from the same generation, weighed before and after the bloodmeal for each assay.

**ELISA.** Ninety-six well plates (Nunc Maxisorb, Nalge Nunc International, Rochester, NY) were coated with the antigen-10  $\mu\text{g/ml}$  chicken-egg albumin (A5503, Sigma-Aldrich) (200  $\mu\text{l/well}$ ) and incubated at  $37^\circ\text{C}$  for 30 min. The solution was removed, and the plates washed three times with PBS-T. PBS (200  $\mu\text{l}$ ) containing 3% nonfat milk was used to block the plates. The plates were frozen with the blocking solution at  $-20^\circ\text{C}$  until used.

Checkerboard titrations were run to determine the optimal working concentrations of the primary and detection antibodies and to develop a reference dilution series to calibrate the assays.

Aliquots of 200  $\mu\text{l}$  of each sample or their controls were added to the plate and incubated for 2 h at room temperature (all samples and controls in triplicate). The plate was then emptied and washed three times with PBS-T.

Goat anti-mouse IgG (whole molecule) antibody, conjugated with alkaline phosphatase (A4656, Sigma-Aldrich) was used as the detector antibody at a 1:1000 dilution. Two hundred microliters of the detector antibody was added to each well, and the plate was incubated for 2 h at room temperature. The plate was emptied and washed three times with PBS-T, and then 200  $\mu\text{l}$  of the enzyme substrate (Sigma Fast *p*-nitrophenyl phosphate tablets in distilled water) was added to each well, and the color was allowed to develop for 30 min. Development was stopped by adding 50  $\mu\text{l}$  of 3 M NaOH to each well, and the plates were read on a Bio-Tek EL340 Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT) at 405 nm. The plate reader was zeroed on the PBS-T blank wells, and the reading from the nonspecific mouse isotype IgG wells

provided a background reading, which was subtracted from the other readings. Based on background readings in preliminary assays, the lower limit for positives was set at optical density (OD) = 0.050; 3 times the background level.

To examine the effect of mAb concentration on subsequent detection, 50 *Ae. aegypti* were fed on one of two concentrations of mAb in blood (1:1000 and 1:10,000 corresponding to 10 and 1  $\mu\text{g}/\text{ml}$ , respectively). After feeding, the mosquitoes were anesthetized with  $\text{CO}_2$ , and blood-fed insects from each treatment were randomly divided into groups of five, placed in mesh covered containers with access to 10% sucrose solution and held for 1, 6, 12, 24, and 48 h at 27°C and 60–70% RH. At the appropriate time, mosquitoes were frozen at  $-80^\circ\text{C}$  until assayed. A nonlinear regression was fitted to the data (DeltaGraph version 5.5.1, Red Rock Software 2005) assuming a constant decay/digestion of mAb within the mosquito, i.e.,  $Y = x e^{-dt}$  where Y is the concentration at time t, x is the concentration at time 0, and d is the decay constant.

To examine the effects of temperature on the putative digestion of mAb and on the duration of detectability after feeding, two groups of mosquitoes were fed mAb in blood and held at two temperatures over selected time intervals. Sixty *Ae. aegypti* were fed on blood containing 1  $\mu\text{g}/\text{ml}$  mAb. Engorged mosquitoes were selected and separated as described above, and groups of five mosquitoes were held for 1, 6, 12, 24, 36, and 48 h at either 27°C and 60–70% RH or at 20°C and 70% RH. At the appropriate times, mosquitoes were frozen at  $-80^\circ\text{C}$  until assayed. As described above, OD was regressed against time using a nonlinear curve fitting routine (DeltaGraph version 5.5.1).

To simulate field studies, we compared storage conditions allowing us to collect and preserve mosquitoes for subsequent analysis. Adult *Ae. aegypti* were fed on blood containing 1  $\mu\text{g}/\text{ml}$  mAb. One hour after feeding, mosquitoes were killed with ethyl acetate, and they were either allowed to air dry at room temperature or they were preserved in 75% ethanol. These mosquitoes were assayed 3 wk later to determine whether the mAb was still detectable.

Again, to simulate field studies and provide proof-of-concept, two blind assays were run to simulate a collection of feral mosquitoes, to determine whether mAb-positive mosquitoes could be identified and if so, to estimate the rate of false positives.

*Ae. aegypti* were fed on 1  $\mu\text{g}/\text{ml}$  concentration of mAb in blood, and they were held at 27°C for 1 h or 24 h, and a control group of *Ae. aegypti* were fed on blood containing a 1:1,000 dilution of the negative control isotype mouse IgG and held for 1 h. All mosquitoes were frozen, and then 12 mosquitoes from each group were numbered and randomized by one of us (C.A.L.). The 36 mosquitoes were assayed blind (by B.J.L.) in an ELISA along with controls and a set of reference dilutions. The mosquitoes were identified as one of two treatment groups or the control, based on their OD readings and comparison to their reference curves and the lower limit. The lower limit for posi-

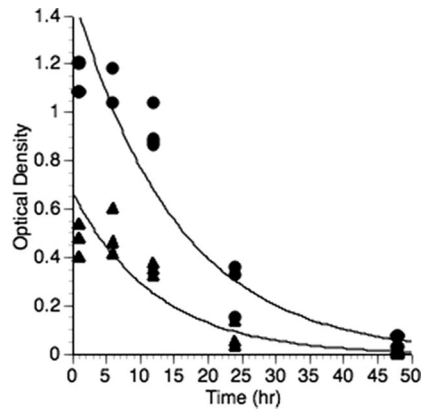


Fig. 1. Degradation of specific antibody (mAb) in blood-fed mosquitoes over time at two different starting concentrations of mAb in blood, 10  $\mu\text{g}/\text{ml}$  (filled circles) and 1  $\mu\text{g}/\text{ml}$  (filled triangles) by using ELISA-based optical density. Curves are from best fit nonlinear regression (DeltaGraph version 5.5.1).

tives was set at OD = 0.05 based on preliminary experiments and the reference curve for the assay.

A second blind assay was run with the same experimental groups with the exception that the number of mosquitoes in each group was unknown. In total, 30 mosquitoes were assayed. The lower limit for positives was set at OD = 0.05.

The ability to detect the presence or absence of mAb in the samples was determined using a chi-square test, where the null hypothesis is 50% success.

## Results

In mosquitoes fed with an initial mAb concentration of 10  $\mu\text{g}/\text{ml}$ , we could detect the antibody in *Ae. aegypti* for 48 h when held at 27°C and 60–70% RH. There was relatively little change in the concentration of mAb detectable during the first 12 h, but there was a rapid decrease in concentration by 24 h, and the concentration approached the lower limit of detection by 48 h. The best fitting line was  $\text{OD} = 1.52 * e^{-0.07 * \text{hr}}$ ,  $r^2 = 0.92$ . This indicates a constant decay (i.e., exponential decline in OD over time) (Fig. 1). Further support for constant exponential decay comes from results from the curve for starting concentration of 1  $\mu\text{g}/\text{ml}$  with best estimates of  $\text{OD} = 0.67 * e^{-0.08 * \text{hr}}$ ,  $r^2 = 0.92$ , wherein the two estimated decay rates are within 14% of one another.

In mosquitoes held at 20°C, we could detect mAb with an initial concentration of 1  $\mu\text{g}/\text{ml}$  for 48 h, although there was a rapid decrease in OD between 24 and 48 h. At 27°C, the same initial concentration of mAb was detected up to 24 h, and the most rapid decrease in OD was between 12 and 24 h. As expected, the decay constant, d, was greater at 27°C. At 20°C, the best fitting line was  $\text{OD} = 0.51 * e^{-0.04 * \text{hr}}$ ,  $r^2 = 0.56$ ; and at 27°C, the best estimates were  $\text{OD} = 0.44 * e^{-0.064 * \text{hr}}$ ,  $r^2 = 0.67$ , i.e., at 27°C, the OD values declined at 1.5 times the rate at 20°C.

After 3 wk at room temperature, the air-dried mosquitoes tested positive, with mean ELISA values of

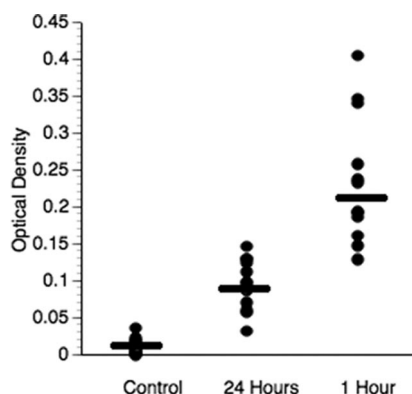


Fig. 2. Blind test 1. *Ae. aegypti* were fed on 1  $\mu\text{g}/\text{ml}$  mAb in blood and held at 27°C after feeding for 1 h ( $n = 12$ ) or 24 h ( $n = 12$ ) and a control group with no mAb in the bloodmeal were held for 1 h ( $n = 12$ ). Mosquitoes were assayed blind and identified to group from OD readings. Lower limit for positives was OD = 0.05. There was one false negative and no false positives. Horizontal bar indicates median.

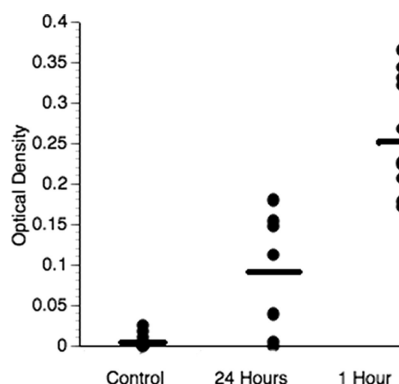


Fig. 3. Blind test 2. Thirty *Ae. aegypti* were fed on 1  $\mu\text{g}/\text{ml}$  mAb in blood and held at 27°C. One group was held for 1 h ( $n = 10$ ), one group for 24 h ( $n = 7$ ), and a control group without mAb in the bloodmeal was held for 1 h ( $n = 13$ ). The number of mosquitoes in each group was unknown during the assay. Mosquitoes were assayed blind and identified to group from OD. Lower limit for positives was OD = 0.05. There were three false negatives and no false positives. Horizontal bar indicates median.

OD =  $0.254 \pm 0.106$  at 405 nm. Mosquitoes that were preserved in 75% ethanol were also positive for mAb after 3 wk, with mean OD readings of  $0.285 \pm 0.130$ . In both treatments, the readings were well above the lower limit of 0.05.

Although there was a considerable variation within each group in the blind tests, it was possible to place nearly all of the mosquitoes in their correct group based on the preset limits. In the first blind test (Fig. 2), the median value for the control was OD = 0.01; for the 24-h group, median value was OD = 0.09; and for the 1-h group, median value was OD = 0.22. There was one false negative ( $\chi^2 = 20.9$ ,  $P < 0.001$ ). In the second blind test (Fig. 3), the median value for the control group was OD = 0.01; for the 24-h group the median was OD = 0.09; and for the 1-h group, median value was OD = 0.27. There were three false negatives ( $\chi^2 = 10.7$ ,  $P < 0.005$ ). False negatives were probably caused by positive group mosquitoes that had taken small bloodmeals. There were no false positives.

### Discussion

Specific IgG could be detected in bloodfed mosquitoes for 24–48 h after feeding by using indirect ELISA. The limits to detection depend on temperature and on the initial concentration of mAb in the mosquito bloodmeal. These initial concentrations of mAb (10 and 1  $\mu\text{g}/\text{ml}$ ) are within the normal range of specific IgG (1  $\mu\text{g}/\text{ml}$ –10  $\text{mg}/\text{ml}$ ) in the blood of seropositive mammals (Harlow and Lane 1999).

*Ae. aegypti* has been used in a number of related studies of antibodies in mosquitoes. It is a tropical mosquito, usually reared at high temperatures (26–29°C). At 27°C, the bloodmeal was rapidly digested, and mAb was detectable only for 24 h at the low mAb concentration (1  $\mu\text{g}/\text{ml}$ ) and 48 h at the higher con-

centration (10  $\mu\text{g}/\text{ml}$ ). The exponential decay curves (Fig. 1) are typical of general protein digestion curves for *Ae. aegypti* at this temperature (Hatfield 1988, Billingsley and Hecker 1991). When *Ae. aegypti* were held at 20°C after feeding, their physical activity and presumably their digestion rate were reduced due to the effects of temperature on the secretion and kinetics of the proteolytic enzymes, and mAb was detectable for 48 h at an initial concentration of 1  $\mu\text{g}/\text{ml}$ .

IgG has been successfully detected in blood-feeding arthropods in other studies (Minoura et al. 1985, Ben-Yakir et al. 1987, Hatfield 1988, Vaughan et al. 1998). IgG is a normal part of the protein component of mosquito bloodmeals, and the putative digestion rate of mAb in this study is typical of other protein digestion studies (Houk and Hardy 1982, Tesh et al. 1988, Irby and Apperson 1989, Billingsley and Hecker 1991). For example, Irby and Apperson (1989) reported that IgG persisted for 36–48 h after feeding in *Ae. aegypti* when mosquitoes were held at 26°C. The use of whole mosquito homogenates in an indirect ELISA would allow for the detection of IgG in midgut or hemolymph (Hatfield 1988), and this method would be most practical in the field.

Edman and Schmid (1970) demonstrated that host blood in mosquitoes stored for periods up to 4 yr could still be identified using precipitin tests. In the current study, we could detect mAb in mosquitoes that were air-dried or alcohol preserved for 3 wk. These methods could be used to preserve and transport mosquitoes from remote areas for antibody studies.

The blind tests simulated the testing of wild-caught mosquitoes and examined the validity of the preset lower limits that allowed for identification of positives with few false negatives and no false positives. The lower limit for positives of OD = 0.05 was based on the limit to detection from the digestion curves. This limit also represents >3 times the highest background level



of the assays in this study. Kemeny (1991) recommended lower limits of 1.5 times the background. Although there were no false positives, the negative controls were as high as  $OD = 0.04$ , so the lower limit of 0.05 may not be adequate. A more appropriate lower limit for this assay would be  $OD = 0.1$ . Chi-square tests indicated that the results of the blind tests were highly significant.

There are many challenges to using this method in the field. The capture of blood-fed mosquitoes in the field is difficult, because such mosquitoes seek out refuges to rest and digest their bloodmeals and they are less likely to be caught in standard mosquito traps. Methods of capturing blood fed mosquitoes are discussed by Leighton (2005).

Collections of blood-fed mosquitoes do not represent random sampling of host animals, because mosquitoes may be differentially attracted to diseased hosts and when mosquitoes take multiple bloodmeals from individuals of the same species or closely related species, it may be difficult to distinguish between host antibodies. If the mixed bloodmeal comes from more distinct taxonomic groups, the detector antibody should distinguish the source of the specific antibodies. The detector antibody could be chosen for the species or general taxonomic group being monitored. Any ELISA system that is developed for use with conventionally collected blood samples from animals could be used with blood-fed mosquitoes.

In field studies, there will always be a chance of having false negatives if mosquitoes contain small bloodmeals or if digestion is too advanced. Selection of well-fed mosquitoes with red, distended abdomens would help to reduce this problem. False positives are a more serious problem, because they could lead to a false diagnosis. The use of standardized positive and negative controls and a predetermined lower limit can reduce the chance of false positives occurring.

It should be possible to use a single blood-fed mosquito for both host antibody analysis and host identification. Service et al. (1986) identified hosts from as little as 0.02  $\mu$ l of blood. In this study, mAb was detectable in whole mosquito homogenates that had been diluted to 200  $\mu$ l, so multiple assays with a single mosquito are possible.

Many techniques have been used to identify the source of the bloodmeal. Serological techniques were reviewed by Washino and Tempelis (1983). Molecular techniques such as polymerase chain reaction (PCR) could provide more specific host identification (Gokool et al. 1993, Boakye et al. 1999, Ngo and Kramer 2003). The amplification of cytochrome *b* gene sequences by PCR and their sequencing showed sufficient interspecific variation to distinguish between mammalian host samples in mosquito bloodmeals (Boakye et al. 1999).

Another type of ELISA that could be useful is an antibody-capture ELISA where an Fc binding protein such as protein A, protein G, or protein L is coated on the plate and binds to sample antibodies (Harlow and Lane 1999). Bound antibodies that are specific to an antigen can be identified by the addition of enzyme-

conjugated antigen. This type of assay does not depend on a specific detector antibody and could be useful in monitoring all the host species in an area for antibodies to a specific pathogen. This method could be useful for seeking reservoir hosts of pathogens. By retaining a portion of each sample, blood from positive results could be used to identify the host species.

As serological techniques become increasingly sensitive, these techniques in combination with quantitative sampling methods could be a useful tool in disease surveillance, for early warning of epizootics, and for studies of immunity in populations, including human populations where normal contact is restricted such as no-contact aboriginal groups or military personnel. Mosquito bloodmeals could possibly be put to other uses such as monitoring environmental toxins in animals or for monitoring other blood components that can be used to determine the nutritional health of animals or to look for signs of noninfectious disease.

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