

## ELISA for Detecting Anti-Tetanus Antibodies in Sumatran Elephants

Lily Natalia<sup>1</sup>, Adin Priadi<sup>1</sup>, Muhammad Wahyu<sup>2</sup>, Anhar Lubis<sup>2</sup> & Christopher Stremme<sup>2\*</sup>

<sup>1</sup>Indonesian Research Centre for Veterinary Science (Balai Besar Penelitian Veteriner), Bogor

<sup>2</sup>Veterinary Society for Sumatran Wildlife Conservation (Vesswic), Medan, Sumatra, Indonesia

\*Corresponding author's e-mail: [stremme@gmx.net](mailto:stremme@gmx.net)

### Introduction

Wounds caused by fights between elephants, foreign bodies, or improper use of management and restraining tools and devices, are a frequent medical issue requiring treatment in captive elephants in Asia (Phuangkum *et al.* 2005; YAGASU 2005; Fowler & Mikota 2006; Fahrimal & Sudarwati 2006). As a result of the above-described wounds, several cases of tetanus from atypical wound infection have been identified in captive elephants in Sumatra during the past years.

Tetanus is a disease that develops if wounds become contaminated with soil or faeces containing *Clostridium tetani* spores. Such wounds become necrotic, are poorly aerated and lack exposure to oxygen, providing optimum conditions for growth of *C. tetani*. After the proliferation of bacteria, 15-20 days later neurotoxins are produced, damaging the nervous system and causing typical muscular spasms.

To prevent the effect of the neurotoxins, treatment with tetanus antitoxin, broad-spectrum antibiotics, re-hydration, and forced feeding over several weeks needs to be implemented. Such treatment is cost- and time-intensive, and often not successful due to delayed identification of the major cause of illness. Furthermore, anaphylactic shock can be a hazard of anti-tetanus therapy because commercially available tetanus antitoxin is a horse serum product. Therefore such treatment is not ideal to address this disease in elephants, and vaccination for prevention would be the ideal. In this study, horse tetanus toxoid vaccines were used to vaccinate Sumatran elephants

Antibody response to antigens of *Mycobacterium tuberculosis*, has been described in elephants

(Lyashchenko *et al.* 2006) and enzyme linked immunosorbent assay (ELISA) has been used to evaluate antibody responses after vaccinations in species other than elephants (Van Weemen & Schuurs 1971; Wright *et al.* 1993; Natalia 1996; Lequin 2005; Van Weemen 2005). But to the best of our knowledge, protection and duration of immunity in elephants after vaccination, has previously been evaluated only in one study with the ELISA method using anti-elephant IgG conjugate (Lindsay *et al.* 2010), and has never been described in Sumatran elephants (*Elephas maximus sumatranus*).

In this preliminary study, an ELISA was developed and used as a monitoring tool to follow the immune responses after tetanus vaccination in groups of Sumatran elephants, with the objective of determining the appropriate dose of tetanus toxoid vaccine.

### Materials and methods

#### 1. ELISA conjugate preparation (anti-elephant IgG labeled horseradish peroxidase)

**Elephant IgG purification:** 50 ml of elephant blood serum was collected from 17 healthy/normal Sumatran elephants. pH of the serum was first adjusted to 8.5, and precipitated with 0.4% rivanol (6,9 Diamino 2 ethoxy acridine lactate monohydrate). 3.5 volumes of rivanol were then added to 1 volume of serum. The supernatant was collected, activated charcoal (1.2 g/100 ml) added and filtered until the filtrate became colourless. One volume of saturated ammonium sulfate was added to 1 volume of supernatant. The precipitate was collected and dialysed against 0.02 M Na<sub>2</sub>HPO<sub>4</sub> pH 7.5 with several changes of buffer solutions. Further purification was done by using DEAE sephacell column chromatography.

IgG/protein estimation was done by the Lowry method.

Immunogen (elephant IgG) preparation and rabbit immunisation: The pure IgG was mixed with Freund's Complete Adjuvant at a ratio of 1:1 and used as prime vaccination. Freund's Incomplete Adjuvant and alum mixtures with IgG were used as boosting vaccine. Immunogen preparation with alum adjuvant was done as follows: 100 ml IgG (2 mg/ml) was added to 50 ml of 1 M NaHCO<sub>3</sub>. Then 100 ml of 0.2 M alumen kalicum was added to the solution and stirred for 30 minutes. The pellets were washed using PBS by centrifuging 3 times at 4000 rpm. The solution was brought back to the original volume and 0.01% merthiolate was added to the alum adjuvanted IgG vaccine.

Four adult male rabbits were used for developing the anti-elephant IgG. The first vaccination was done by injecting 0.5 ml IgG + Freund's Complete Adjuvant (FCA)/s.c. Four weeks later, revaccination was done by injecting the same dose of IgG + Freund's Incomplete Adjuvant (FIA)/s.c. At 7 weeks after the first vaccination, 0.5 ml alum adjuvanted IgG was injected in four sites intramuscularly in each rabbit. The rabbits were vaccinated with alum adjuvanted IgG 4 times at one-week intervals. Two weeks after the last immunization, all rabbits were bled and sera were collected.

Anti-elephant IgG horseradish peroxidase labelling: Anti-elephant IgG was then purified following methodology already described (Johnstone & Thorpe 1982; Akita & Nakai 1993; Cadman *et al.* 1993), and labeled with horseradish peroxidase following methodology described by Nakane & Kawoi (1974).

The conjugate was stored at 4°C until use. Specificity of the conjugate was tested using dot-blot assay. Two µl each of elephant, bovine, buffalo, horse, sheep, and ostrich serum were dotted onto nitrocellulose membrane. The membrane was air dried and blocked with PBS-tween containing 0.2% casein for 2 hours. After washing with PBS-tween three times, the membrane was embedded in PBS-T-Casein

0.2% containing anti-elephant conjugate diluted 1:200. The membrane was washed after a 1 hour incubation. Colour development was visualised by adding substrate solution containing 3,3-diaminobenzidine 6 mg, 20 µl H<sub>2</sub>O<sub>2</sub> in 10 ml citrate buffer pH 4.2.

## 2. Preparation of tetanus ELISA coating antigen

Toxin was prepared by using *Clostridium tetani* strain ATCC 8033. Culture was grown in broth containing 3% proteose peptone and 0.1% NaCl. 0.2 g iron was added to 1 l of broth. The alternative media for toxin production is media of LATHAM (1962), see Table 1. These ingredients were made up for 1 l of media. Starter culture was made by inoculating Robertson's Cooked Meat Broth with freeze-dried *C. tetani*. The culture was incubated at 37°C for 3 days. Toxin produced was centrifuged at 8000 RPM and sterile filtered. Tetanus toxoid was obtained by adding 0.3% formalin and incubated at 37°C for 3 weeks. Toxoid was purified by adding 50% saturated ammonium sulphate. The precipitate formed was dialysed against PBS. Toxoid was further purified by DEAE Sephacel column. After purification, the toxoid was freeze-dried, and was ready to be used as the ELISA coating antigen.

## 3. Preparing negative and positive control sera

Elephants were vaccinated with equine tetanus toxoid (Intervet Inc. Milsboro) at a dose of 1 ml intramuscularly (i.m.). They were revaccinated several times at monthly intervals with the same dose until the elephant hyperimmune serum was obtained. This hyperimmune serum is used as positive control in ELISA. To obtain ELISA negative control serum, some unvaccinated normal/healthy elephants were bled and the serum was screened.

## 4. ELISA standardization for anti-tetanus toxin detection

For obtaining optimal working dilutions, coating antigen (tetanus toxoid), serum, and anti-elephant conjugate were titrated using the checkerboard method. Negative and positive control elephant sera were also included in the ELISA. The

assay was carried out using 96 well Ubottom microplates (Nunc, Maxisorp) as a solid phase device. Purified tetanus toxoid was diluted 1/200 in carbonate buffer pH 9.6 and used as a coating antigen. Coating was done overnight at 4°C. The microplate was washed three times using Phosphate Buffered Saline (PBS) containing 0.05% Tween 20 (PBST). Blocking was done overnight using PBS containing 0.2% casein. Serum samples were diluted 1/20 in PBST and added to microplate wells at a volume of 100  $\mu$ l. After shaking at room temperature for 1 hour, the microplate was washed three times using PBST. Anti-elephant IgG in 1/500 dilution was added to the wells, and shaken for another hour at room temperature. After three washings, 2,2-Azino bis (3-ethylbenzothiazoline-6-sulfonic acid) di ammonium salt (ABTS, Sigma) was added and left for an hour. The optical density of each well was read by using an ELISA reader (Multiskan EX) at 414 nm wavelength.

### 5. Vaccination evaluation

Four groups of elephants of an estimated age range of 12 to 25 years (5 males, 12 females) were vaccinated with different doses of tetanus toxoid. Tetanus toxoid (Intervet, Milsboro, equine vaccine) was given at a dose of 1 ml, 2 ml, 3 ml, and 4 ml i.m. for each group respectively. Because this study was conducted as part of a routine preventative vaccination program, the majority of the elephants in group 1 (11 elephants) were vaccinated with the 1 ml dose which is the elephant dose routinely described (Fowler & Mikota 2006). There was no existing data about the post vaccination antibody titers at the time the study was started, and the data from Lindsay *et al.* (2010) was not yet published. Groups 2, 3, and 4 each consisted of 2 elephants. The elephants in these four groups had not previously received any kind of vaccination. The initial vaccination was followed by a booster after 4 weeks. One blood sample was collected immediately before the first vaccination, then 4 weeks later a sample was collected immediately before the booster, followed by samples at 1-month intervals for one year to monitor the immune response over this period. The collected serum samples were then tested with the standardized anti-tetanus ELISA.

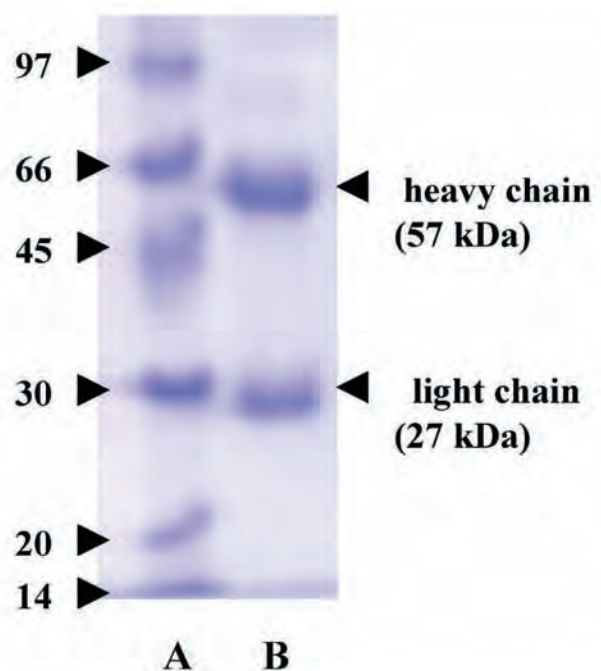
## Results

### *Elephant IgG purification*

Elephant serum/blood was collected in a captive elephant camp in North Sumatra from a total of 17 elephants. Fifty ml of elephant serum was sent to BALITVET in Bogor/Java in September 2006 for development of anti elephant IgG conjugate. Visually the viscosity of the elephant serum seemed to be lower compared to sera of several livestock species such as cattle, goat, buffalo, and sheep. Four (4) ml of purified IgG at a concentration of 9.718 mg/ml was obtained from 50 ml of elephant serum. Protein profile of the purified elephant IgG in Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) is shown in figure 1. Two protein bands of approximate molecular weight of 57 kDa and 27 kDa were shown in SDS PAGE electrophoresis.

### *Preparation of ELISA conjugate (anti-elephant IgG labeled horseradish peroxidase)*

Rabbits responded very well to elephant IgG, resulting in rabbit anti-elephant IgG in a concentration of 48.83 mg/ml. Conjugate obtained



**Figure 1.** SDS PAGE analysis. Lane A: Molecular standard weight. Lane B: heavy chain and light chain of purified elephant IgG.

from conjugation of the anti-elephant IgG with horseradish peroxidase showed a high specificity to elephant IgG as shown by dot blot assay (Fig. 2). It did not react to bovine, buffalo, sheep, and ostrich immunoglobulins. A very mild reaction of the conjugate to horse Ig was observed.

#### *ELISA for tetanus antitoxin detection*

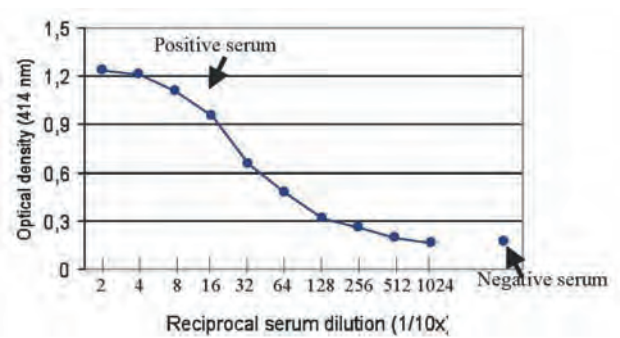
From the checkerboard titration, a standard curve from positive serum was obtained with coating antigen at a 1:200 dilution and conjugate at a 1:500 dilution (Fig. 3). The end point of the positive serum was achieved at a 1:10,240 dilution. At this end point the optical density value of the positive serum was equal to the negative serum at a 1:20 dilution; this dilution was used in the entire study.

#### *Immune response evaluations after tetanus vaccination in 4 groups of elephants*

The results of ELISA, which were interpreted in the sample to positive ratio (SP ratio) in elephant serum samples taken monthly for a 1-year period, can be seen in the antibody profile from the 12 months of monitoring shown in figure 4.

#### **Discussion**

The concentration of 9.718 mg/ml obtained from the elephant serum in this study is lower when compared to rabbit IgG which contained a concentration of 48.83 mg/ml. The heavy chain of 57 kDa detected from elephant immunoglobulin is lower than the heavy chain reported in other species, which consists of 67.5 kDa molecules. The 27 kDa component is analogous to the light chains in other species (Cadman et al. 1993). Unlike serum dilution in ELISA of other

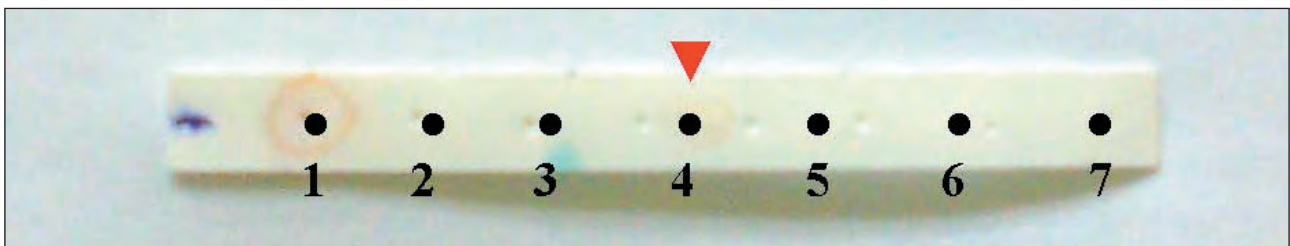


**Figure 3.** ELISA standard curve for detecting anti-tetanus in elephant sera.

species which uses dilutions of 1:100 to 1:500, in elephants, the serum dilution was 1:20. The lower dilution may have been due to the low content of protein in the elephant serum.

The data in figure 1 and figure 2 shows that rabbits gave a good response after immunization with purified elephant IgG. In dot-blot assay using rabbit anti-elephant IgG labeled with horseradish peroxidase enzyme blotted to elephant, bovine, buffalo, sheep, and ostrich immunoglobulins, reaction was only observed in elephant serum. However, this conjugate reacted very weakly with horse serum (Fig. 2). This specific rabbit anti-elephant IgG conjugate will be a valuable tool for the development of immunoassays to detect specific antibodies against diseases in elephants.

Using a checkerboard titration, the positive serum gave an end point of 1:10,240 dilution in the newly developed ELISA. To apply the ELISA for serological monitoring of tetanus vaccination, the results were interpreted in the sample to positive ratio (SP ratio). The SP ratio has the benefit of normalizing results under a variety of conditions. In this case, both test controls and serum samples are subject to the same conditions, so the SP ratio cancels out the effect of all external factors that



**Figure 2.** Reactions of rabbit anti-elephant horseradish peroxidase conjugate to elephant (1), bovine (2), buffalo (3), horse (4), sheep (5), ostrich (6) immunoglobulins and saline (7) in dot-blot assay. A very mild reaction of the conjugate to horse Ig was observed (arrow).

affect the entire plate (temperature, incubation time, reagent concentrations, etc.). This enables specific antibodies in the sample to be accurately and reproducibly measured.

In general, peak antibody response to vaccination occurred 1 month after the booster vaccination. After this peak, the antibody declined gradually until the end of observation. Nearly all of the elephants reached the antibody peak one month after booster vaccinations were given, with the exception of 2 animals (Esther and Edi) whose samples showed the peak was reached one month after the initial vaccination.

The immune response shown by Edi was very low. Edi was an elephant captured several months prior to the vaccination, in poor body condition, and still recovering from the capture and initial training procedures, which seems likely to have negatively affected the immune response of this

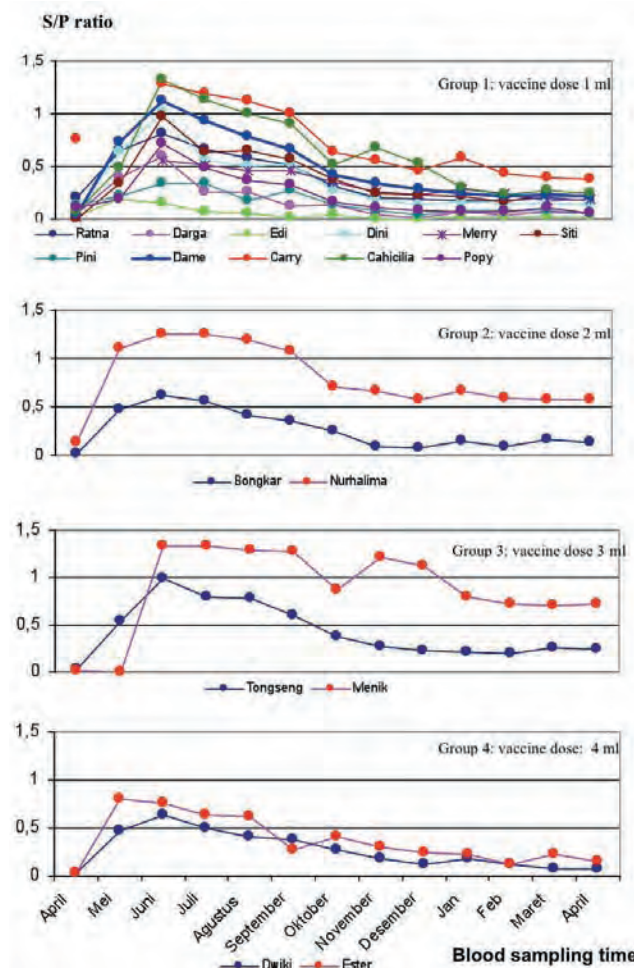
individual. The serological titers then declined until the end of observation in April 2009.

Due to unequal numbers of serum samples from each group of elephants, and low numbers of individuals in the groups 2, 3 and 4, the interpretation of the best immune responses was unclear. The increasing dose of tetanus toxoid did not appear to give a correspondingly increasing immune response. A trend of possible increase with a 2-3 ml vaccine and a decrease with a 4 ml vaccine was noted. Determination of the appropriate dose of tetanus toxoid for elephants should be further investigated by using a larger number of elephants per sample group. It would be ideal if the elephants used in the study are of the same age, body weight, and managed in the same manner and in a similar environment.

The variation of immune response in group 1 (vaccinated with 1 ml of tetanus toxoid) was high. In serological observation, high uniformity of antibody titer is an important factor for determining immune responses. Therefore investigating immune response variation with a larger sample size for higher vaccine doses is indicated.

The ELISA developed in this study would be useful as a component for sero-diagnosis and sero-monitoring tests for diseases in elephants. In the ELISA study by Lindsay *et al.* (2010), a double sandwich indirect ELISA was used. For the Sumatra study we used indirect ELISA where the rabbit anti-elephant IgG was directly labeled with horseradish peroxidase. For both methods the sensitivity is likely to be the same, but the method presented here takes less time and is probably more cost efficient.

In our study the average age of the elephants was younger than in the Lindsay *et al.* (2010) study where the elephants very divided into 3 different age classes and the same dosage of tetanus vaccine used in all animals, whereas in our study different dosages of vaccine were used. Elephants used in our study were vaccinated for the first time ever with tetanus vaccine, and were given a booster after the initial dose. The Lindsay *et al.* (2010) study used elephants that had already been



**Figure 4.** Immune responses in elephants vaccinated in April and again in May 2008 detected by ELISA.

vaccinated some years prior to the study and were only vaccinated with a single dose. Both studies show a clear post vaccination antibody response, but in our study in the majority of the elephants (all except 2) the peak antibody response was reached 30 days after the second and 60 days after the first vaccination. In the Lindsay *et al.* (2010) study, using only a single vaccination the peak antibody response in the majority of the elephants was reached 30 days after this single vaccination.

## Conclusion

We successfully developed an ELISA for the detection of anti-tetanus antibodies in Sumatran elephants. DEAE Sephacel purified tetanus toxoid can be used as a coating antigen. Horseradish peroxidase labeled anti-elephant IgG conjugate developed in rabbits differentiates positive serum and negative serum very well. Sero-monitoring of different tetanus vaccination doses using ELISA gave a good profile of antibody responses in elephants.

Using commercially available tetanus toxoid vaccines in elephants resulted in a clear antibody response and may provide effective protection against tetanus infection. Further studies are needed using a larger and more uniform elephant population sample set to validate higher antibody response and uniformity noted with vaccine dosages of 2 and 3 ml, and thus determine the most effective dosage of vaccine as well as ideal re-vaccination intervals.

## References

- Akita EM & Nakai S (1993) Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. *Journal of Immunological Methods* **160**: 207-214.
- Cadman HF, Kelly PJ, Dikanifura M, Carter SD, Azwal SM & Wright EP (1993) Isolation and characterization of serum immunoglobulin classes of the ostrich (*Struthio camelus*). *Avian Diseases* **38**: 616-620.
- Fowler ME & Mikota SK (2006) *Biology, Medicine, and Surgery of Elephants*. Blackwell Publishing Professional, Ames, Iowa, USA.
- Fahrimal Y & Sudarwati R (2006) Veterinary problems of geographical concern. Section IV Indonesia In: *Biology, Medicine, and Surgery of Elephants*. Fowler ME & Mikota SK (eds) Blackwell Publishing, Oxford, UK. pp 454-457.
- Johnstone A & Thorpe R (1982) *Immunochemistry in Practice*. Blackwell Scientific Publications, Oxford. pp 41-56.
- Lequin RM (2005) Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical Chemistry* **51**: 2415-2418.
- Lindsay WA, Wiedner E, Isaza R, Townsend HGG, Boleslawski M & Lunn DP (2010) Immune response of Asian Elephants (*Elephas maximus*) to commercial tetanus toxoid vaccine. *Veterinary Immunology and Immunopathol.* **133**: 287-289.
- Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**: 265-275.
- Lyashchenko KP, Greenwald R, Esfandiari J, Olsen JH, Ball R, Dumonceaux G, Dunker F, Buckley C, Richard M, Murray S, Payeur JB, Andersen P, Pollock JM, Mikota S, Miller M, Sofranko D & Waters WR (2006) Tuberculosis in elephants: Antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. *Clinical and Vaccine Immunology* **13**: 722-732.
- McCullough KC (1993) The application of biotechnology to the diagnosis and control of Animal diseases. *Revue Scientifique et Technique, International Office of Epizootics* **12**: 325-353.
- Natalia L (2000) Manifestasi radang paha pada hewan. *JITV* **5**: 53-58.
- Natalia L (1997) Pengembangan teknik ELISA untuk mendeteksi toksin alfa *Clostridium novyi*. *JITV* **2**: 188-194.

Natalia L (1996) Evaluasi respon antibodi sapi dan kerbau terhadap vaksin *Clostridium perfringens* tipe A dengan menggunakan ELISA. *JITV* **1**: 190-193.

Nakane PK & Kawoi A (1974) Peroxidase labeled antibody: a new method of conjugation. *Journal of Histochem. and Cytochemistry* **22**: 1084-91.

Phuangkum P, Lair RC & Angkawanith T (2005) *Elephant Care Manual for Mahouts and Camp Managers*. Forest Industry Organization, Ministry of Natural Resources and Environment, Food and Agriculture Organization of the United Nations, Regional Office for Asia and the Pacific.

Van Weemen BK (2005) The rise of EIA/ELISA. *Clinical Chemistry* **51**: 2226.

Van Weemen BK & Schuurs AHWM (1971) Immunoassay using antigen-enzyme conjugates. *FEBS Letters* **15**: 232-236.

Wright PF, Nilsson EP, Van Rooij EMA, Lelenta M & Jeggo MH (1993) Standardization and validation of enzyme linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Revue Scientifique et Technique, International Office of Epizootics* **12**: 435-450.

YAGASU (Yayasan Gajah Sumatera) (2005) *Veterinary Care Support Program*. Annual Report 2005.



Group in Udawalawe National Park (Sri Lanka)  
Photo by Prithiviraj Fernando