

Development and evaluation of an interferon- γ release assay in Asian elephants (*Elephas maximus*)

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ABSTRACT. We developed an interferon- γ release assay (IGRA) specific for Asian elephants (*Elephas maximus*). Whole blood collected from forty captive Asian elephants was stimulated with three different mitogens i.e., phytohemagglutinin (PHA), pokweed mitogen (PWM) and phorbol myristate acetate/ionomycin (PMA/I). A sandwich ELISA that was able to recognize the recombinant elephant interferon- γ (rEIFN- γ) as well as native interferon- γ from the Asian elephants was performed using anti-elephant IFN- γ rabbit polyclonal antibodies as capture antibodies and biotinylated anti-elephant IFN- γ rabbit polyclonal antibodies as detection antibodies. PMA/I was the best mitogen to use as a positive control for an Asian elephant IGRA. The development of an Asian elephant-specific IGRA that detects native IFN- γ in elephant whole blood provides promising results for its application as a potential diagnostic tool for diseases, such as tuberculosis (TB) in Asian elephants.

KEY WORDS: Asian elephant, interferon- γ , mitogen, sandwich ELISA

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Asian elephant (*Elephas maximus*) is an endangered species according to The IUCN Red List of Threatened Species [2]. There are approximately 208 captive Asian elephants in Nepal which are primarily used for patrolling protected areas, in eco-tourism, and for wildlife research projects [12]. Asian elephants are susceptible to many infectious diseases, and currently infection by intracellular pathogens like *M. tuberculosis* [8, 11] and elephant endotheliotropic herpesviruses [13] are increasingly seen.

A cell-mediated immune response is elicited by the body during the early stages of infection by intracellular pathogens including *M. tuberculosis*. Interferon- γ (IFN- γ) is one

of the major cytokines secreted mainly by Th₁ and Natural Killer cells. Mediated by the up regulation of reactive oxygen intermediates and toxic nitric oxide (NO), IFN- γ causes the activation of macrophages that are programmed to kill obligate intracellular microbes [3]. Cell-mediated immunity is activated earlier than humoral immunity. IFN- γ plays an important role in the immuno-pathogenesis of tuberculosis (TB) [5]. Bovigam (Prionics AG, Zurich, Switzerland) was the first commercial IFN- γ release assay (IGRA) developed for the diagnosis of TB in bovine species. Quantiferon Gold-In-Tube (Cellestis Inc., Melbourne, Australia) was later developed as an IGRA for TB diagnosis in humans. Recently, IGRAs have also been developed in some wildlife species including deer species [14], lions [7] and white rhinoceros [9] for the diagnosis of TB.

The purpose of this study was to develop and evaluate an Asian elephant-specific IGRA and see its potential as a diagnostic tool for diseases, such as TB in this species.

MATERIALS AND METHODS

Expression and purification of recombinant Asian elephant interferon- γ (rEIFN- γ): The elephant interferon- γ (EIFN- γ) gene (458 bp) was synthesized and cloned into *Nde* I and *Eco*RI sites of pET-17b vector (Merck KGaA, Darmstadt,

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Germany). *E. coli* SoluBL21 (DE3) (Merck KGaA) harboring the EIFN- γ expression vector was grown in LB broth in the presence of 100 $\mu\text{g/ml}$ of carbenicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at 37°C. The recombinant protein expression was induced by the addition of isopropylthiogalactoside (IPTG) (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) to a final concentration of 0.1 mM followed by incubation at 16°C for 22 hr. The *E. coli* cells were resuspended in 40 mM Tris-HCl (pH 8), 0.15 M NaCl and 10% (w/v) sucrose containing EDTA-free complete (Roche Applied Science, Penzberg, Germany) and disrupted using a sonicator, and the cell debris was removed by centrifugation at $140,000 \times g$ for 120 min. All subsequent purification steps were performed at 4°C. The supernatant was mixed with TALON[®] resin (Takara Bio Inc., Otsu, Japan), and the resin was washed with 20 mM TrisHCl pH 8, 0.15 M NaCl, 5 mM Imidazole (Wako Pure Chemicals Industries, Ltd.). The recombinant EIFN- γ was eluted from the resin with 20 mM Tris-HCl pH 8, 0.15 M NaCl, 0.2 M Imidazole by a batch method and dialyzed against 20 mM Tris-HCl pH 8.8, 0.1 M NaCl, 3% glycerol (v/v) and 0.1 mM EDTA overnight and applied onto a 2.6×10 cm diethylaminoethanol fast flow (DEAE FF) column (GE Healthcare, Little Chalfont, U.K.). The protein was eluted with a 10 column volume (CV) of linear gradient (0.1 to 0.6 M NaCl). The eluted protein was concentrated, loaded onto a 1.6×60 cm Superdex 75 prep grade column (GE Healthcare) and eluted with 20 mM Tris-HCl pH 8.8, 0.2 M NaCl, 3% glycerol (v/v) and 0.1 mM EDTA. The eluted protein was dialyzed against 20 mM Tris-HCl pH 8.8, 0.15 M NaCl, 20% glycerol (v/v) and 0.1 mM EDTA, concentrated and stored at -83°C.

Production, purification and labeling of polyclonal antibodies: The anti-EIFN- γ polyclonal antibodies were produced by immunization of recombinant rEIFN- γ in rabbits. Antibodies were incubated for 9 weeks, then the rabbits were bled, and the serum was collected. Purification of antibodies was performed by affinity chromatography using protein A agarose (GE Healthcare) according to the manufacturer's protocol.

The purified antibodies were used as capture antibodies. For the purpose of producing detection antibodies, these antibodies were biotinylated using Biotin Labeling Kit-NH₂ (Dojindo Chemical Co., Ltd., Kumamoto, Japan) as per the manufacturer's instruction. Briefly, affinity-purified rabbit polyclonal antibody against rEIFN- γ with a recommended concentration between 50–200 μg was mixed with recovery buffer in a filtration tube and centrifuged at $8,000 \times g$ for 10 min. Then, reaction buffer and NH₂-reaction biotin solution were mixed in the same tube and incubated for 10 min at 37°C. Unlabeled antibodies were washed by adding recovery buffer followed by centrifugation at $8,000 \times g$ twice. Finally, 200 μl of recovery buffer was added to the tube and mixed thoroughly to recover the conjugate, then transferred to a sterile tube and stored at 4°C until use.

Determination of optimum conditions for coating and detection antibodies: We performed a sandwich ELISA to determine the optimum conditions for coating and detection of antibodies. A flat-bottomed polystyrene microtiter plate

(Nunc A/S, Roskilde, Denmark) was coated with variable dilutions of unlabeled anti-EIFN- γ polyclonal antibodies (0.1, 0.3, 1 and 2 $\mu\text{g/ml}$) and incubated at 4°C overnight. The plate was washed three times with phosphate-buffered saline (PBS, pH 7.6) containing 0.05% Tween-20 (PBST) and incubated with a blocking solution containing 3% (w/v) bovine serum albumin (Roche Dx, Mannheim, Germany) for 1 hr at 37°C and washed again as described earlier. Various dilutions of rEIFN- γ (1, 3, 10, 30, 100, 300 and 1,000 $\mu\text{g/ml}$) were added to the wells. After incubation for 1 hr at 37°C, the plate was washed three times with PBST, variable dilutions of biotin-labeled anti-EIFN- γ polyclonal antibodies (0.1, 0.3, 1 and 3 $\mu\text{g/ml}$) were added to the wells, and the plate was incubated for 1 hr at 37°C. Three washes using PBST were performed, then 100 μl of avidin-biotin complex stain (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) was added, and the plate was incubated at room temperature for 30 min. Finally, three additional PBST washes were performed, then 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) was added to each well, and the plate was incubated at room temperature for 10 min. The reaction was stopped by adding 1M phosphoric acid (Wako Pure Chemicals Industries, Ltd.). The absorbance value of each well was measured at 450 nm using a microplate reader.

Selection of study subjects: Forty captive Asian elephants were selected for the study from three protected areas of Nepal. Thirty four elephants were females, and 6 were males; age ranged from 8–72 years. Eight elephants were from Bardia National Park, six from Parsa Wildlife Reserve, and 26 were from Chitwan National Park. None of the elephants had positive *Mycobacteria* cultures at the time of blood collection.

Blood collection, stimulation and incubation: A whole blood sample was collected from the auricular vein of each elephant into a Mitogen (Phytohemagglutinin) (Quantiferon Gold in-Tubes; Cellestis Ltd.) and a heparinized tube. One ml of blood was collected in the Mitogen tube, and approximately 3 ml of blood was collected in the heparin tube. The tubes were shaken gently 10 times after blood collection. One ml blood from the heparin tube was put into each of two tubes containing Pokweed mitogen (PWM) and Phorbol myristate acetate and Ionomycin (PMA/I) (Sigma Aldrich, Zwijndrecht, The Netherlands), respectively. These two tubes were also shaken gently 10 times after filling them with the heparinized blood. Different concentrations of PWM (5 $\mu\text{g/ml}$), PMA/I (100 ng/ml) and Ionomycin (2 $\mu\text{g/ml}$) were used. The incubation of the whole blood tubes was performed at 37°C for 16–24 hr in the field before transporting the samples to the laboratory in Kathmandu for ELISA. The blood tubes were then centrifuged, and the supernatant was harvested and subjected to a sandwich ELISA.

Sandwich ELISA: An ELISA plate containing 96 wells was coated with capture antibodies, and then, 50 μl of anti-elephant IFN- γ rabbit polyclonal antibodies diluted with 1 x PBS at 1 $\mu\text{g/ml}$ was placed in each well. The capture antibodies were incubated for 1 hr at 37°C. The plates were washed manually three times at room temperature with PBST. The

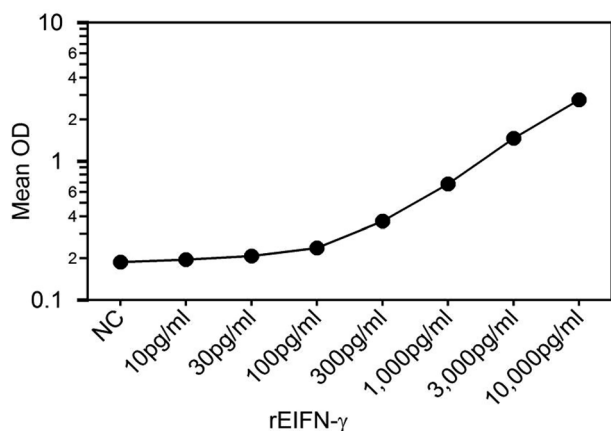


Fig. 1. Titration curve of recombinant elephant IFN- γ (rEIFN- γ) optimized by using coating antibodies (1 $\mu\text{g/ml}$) & detection antibodies (3 $\mu\text{g/ml}$). NC=Negative Control with 0 pg/ml rEIFN- γ .

reaction was blocked with 50 μl per well of blocking buffer for 1 hr at 37°C. The plates were washed with PBST three times, and then, 50 μl of elephant plasma was placed in each appropriate well and incubated for 1 hr at 37°C. The ELISA buffer was run as a negative control, and the rEIFN- γ was run in duplicate in various concentrations (10, 30, 100, 300, 1,000, 3,000 and 10,000 pg/ml) as the positive control for each plate. The plates were again washed three times with PBST. Biotin-labeled anti-elephant IFN- γ rabbit polyclonal antibodies (3 $\mu\text{g/ml}$) were diluted with ELISA buffer at 1:10,000, and 50 μl of this diluted antibody was placed into each well and incubated for 1 hr at 37°C. After incubation, the plates were washed three times with PBST, then 100 μl of avidin-biotin complex stain was added to each well, and the plate was incubated at room temperature for 30 min. Washing was performed three times using PBST, and 100 μl of TMB substrate was placed in each well for 10 min. The reaction was stopped using 100 μl stop solution, and the optical density was measured at a wavelength of 450 nm. Optical density values were converted to IU/ml values, and finally, each IU/ml value was converted to pg/ml by multiplying each IU/ml value by 40 pg/IU [4].

RESULTS

The optimum combination of capture and detection antibodies from sandwich ELISA was obtained at 1 $\mu\text{g/ml}$ of capture antibodies and 3 $\mu\text{g/ml}$ of detection antibodies. The lowest limit of detection (LOD) of rEIFN- γ in this optimized sandwich ELISA was 100 pg/ml (Fig. 1).

The sandwich ELISA system developed was able to detect rEIFN- γ as well as native interferon- γ from Asian elephants as elicited by stimulation using PMA/I, PWM and PHA (Fig. 2). Of the three mitogens, stimulation by PMA/I was highest followed by PWM and PHA (Fig. 2). Thus, PMA/I is the best mitogen to use as a positive control for an Asian elephant IGRA. The statistical analysis also showed that there was no significant difference in the values of the three

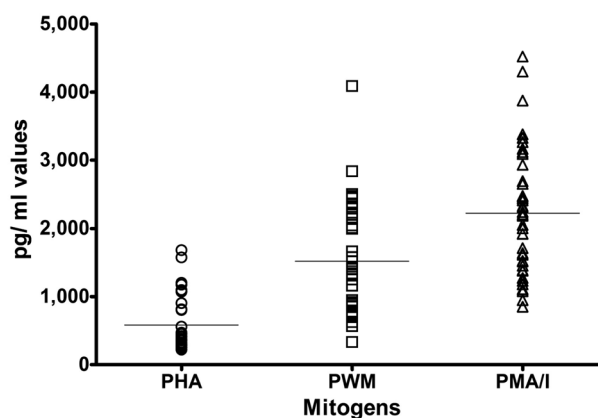


Fig. 2. Stimulation of elephant plasma by three mitogens i.e., phytohemagglutinin (PHA), pokweed mitogen (PWM) and phorbol myristate acetate/ionomycin (PMA/I).

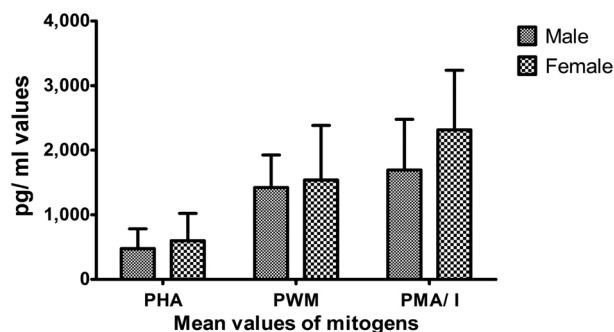


Fig. 3. Comparison of three mitogen values i.e., phytohemagglutinin (PHA), pokweed mitogen (PWM) and phorbol myristate acetate/ionomycin (PMA/I) between male and female Asian elephants of Nepal.

mitogens based on gender (Fig. 3).

DISCUSSION

This study describes the expression and purification of rEIFN- γ , production of polyclonal antibodies specific for rEIFN- γ and optimization of a sandwich ELISA. The LOD of the ELISA system was determined to be 100 pg/ml (Fig. 1). Similar detection levels have also been obtained in lions [7]. Thus, the sensitivity of our sandwich ELISA was sufficient to detect native IFN- γ in elephants as elicited by mitogenic stimulation (Fig. 2).

IGRA performed showed that all the elephants were able to produce detectable levels of IFN- γ following stimulation with the three different mitogens. Among the mitogens, PMA/I was recognized as the best mitogen to be used as a positive control for the stimulation of the Asian elephant whole blood followed by PWM (Fig. 2). Thus, this study shows that PMA/I was the best mitogen in Asian elephants, however, the reason behind this could not be established.

PMA/I has previously been identified as the best performing mitogen in Asian elephants [1] and white rhinoceros [10].

Culture of trunk wash samples is considered the gold standard for TB diagnosis in elephants; however, this technique has several limitations including difficulty of sample collection, a long culture period and poor sensitivity [6]. The ELISA system developed in this study could be performed by incorporating ESAT-6/CFP-10 as a fusion protein for the whole blood stimulation for early diagnosis of TB in elephants in the future. If this assay is validated in Asian elephants, it will be very useful for early diagnosis of TB which will eventually offer several advantages over culture including the easiness of sample collection and getting the results within a couple of days.

In a study conducted in Thailand, IGRA was performed on four Asian elephants for diagnosis of TB among which one was found to be positive with *M. tuberculosis* in trunk wash culture [1]. However, none of the elephants used in this study were culture positive for TB. The study from Thailand used the monoclonal antibodies for capture and detection antibodies; however, we used polyclonal antibodies in the assay. The LOD of IGRA was lower in Thai elephants than that in our study. Such discrepancies could be attributed to geographical locations, variation in sample size and use of different antibodies for ELISA.

As female elephants are more docile and co-operative, they are widely used as working elephants. Not many studies have been done in humans to compare the IFN- γ values between males and females. Female elephants are more frequently used for safaris and other activities that provide them greater chances of contracting TB from the infected human patient and carry the infection for longer time without showing any signs and symptoms. Due to this reason, the cows might show higher mitogenic stimulation of lymphocytes to produce IFN- γ than the bulls. However, we did not find any significant differences in the lymphocyte stimulation by three mitogens between male and female elephants (Fig. 3).

In conclusion, the development of an Asian elephant-specific IGRA that detects native IFN- γ in elephant whole blood provides promising results for application as a potential diagnostic tool for diseases, such as TB in Asian elephants. An IGRA study in the future using a larger population of Asian elephants and including TB-specific antigens might help to diagnose TB in the early stages of the disease.

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