The elephant interferon gamma assay: a contribution to diagnosis of tuberculosis in elephants

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Summary

Mycobacterium tuberculosis (*M. tb*) has been shown to be the main causative agent of tuberculosis in elephants worldwide. *M. tb* may be transmitted from infected humans to other species including elephants and *vice versa*, in case of prolonged intensive contact. An accurate diagnostic approach covering all phases of the infection in elephants is required. As *M. tb* is an intracellular pathogen and cell-mediated immune (CMI) responses are elicited early after infection, the skin test is the CMI assay of choice in humans and cattle. However, this test is not applicable in elephants. The interferon gamma (IFN-γ) assay is considered a good alternative for the skin test in general, validated for use in cattle and humans. This study was aimed at development of an IFN-γ assay applicable for diagnosis of tuberculosis in elephants. Recombinant elephant *IFN-γ* (*rEpIFN-γ*) produced in eukaryotic cells was used to immunize mice and generate the monoclonal antibodies. Hybridomas were screened for *IFN-γ*-specific monoclonal antibody production and subcloned, and antibodies were isotyped and affinity purified. Western blot confirmed recognition of the *rEpIFN-γ*. The optimal combination of capture and detection antibodies selected was able to detect *rEpIFN-γ* in concentrations as low as 1 pg/ml. The assay was shown to be able to detect the native elephant IFN-γ, elicited in positive-control cultures (pokeweed mitogen (PWM), phorbol myristate acetate plus ionomycin (PMA/I)) of both Asian and African elephant whole-blood cultures (WBC). Preliminary data were generated using WBC from non-infected elephants, a *M. tb* infection-suspected elephant and a culture-confirmed *M. tb*-infected elephant. The latter showed measurable production of IFN-γ after stimulation with ESAT6/CFP10 PPDB and PPDA in concentration ranges as elicited in WBC by *Mycobacterium tuberculosis* complex (MTBC)-specific antigens in other species. Hence, the *IFN*-γ assay presented potential as a

diagnostic tool for the detection of elephant tuberculosis. Validation of the assay will require its application in large populations of non-infected and infected elephants.

Introduction

For thousands of years elephants have been trained for the purpose of human use. As a result, especially captive Asian elephants (*Elephas maximus*) are often in prolonged contact with humans and are prone to infection with *Mycobacterium tuberculosis* (*M. tb*), as first reported in 1875 (Michalak et al., 1998). *M. tb* may be transmitted from infected humans to other species including elephants and *vice versa*, in case of prolonged intensive contact (Une and Mori, 2007, Angkawanish et al., 2010 and Murphee et al., 2011). Infection has an impact on animal welfare and constitutes a risk for humans, hence for the tourist industry and may have economical consequences. In the last two decades elephant tuberculosis has been reported from all around the world (Michalak et al., 1998, Mikota et al., 2001, Payer et al., 2002, Oh etal., 2002, Lewerin et al., 2005, Une and Mori, 2007, Angkawanish et al., 2010 and Murphee et al., 2011), hence an accurate diagnostic approach covering all phases of the infection is required. The gold standard being bacterial culture from trunk washes and affected tissues at necropsy is insensitive (Mikota et al., 2001 and Lyaschenko et al., 2006). Diagnostic assays detecting antibodies specific for *M. tuberculosis* Complex (MTBC) antigens in serum have their limitations, especially due to cross-reactivity caused by non-pathogenic mycobacteria (Greenwald et al., 2009). Moreover, they do not form part of the initial immune responsiveness. Since *M. tb* is an intracellular pathogen, cell mediated immune (CMI) responses shown to be elicited early after infection are considered to contribute to protection. The skin test, the CMI assay of choice for diagnosis of tuberculosis in humans and cattle, is not applicable in pachyderm species. However, the interferon gamma (IFN-γ) assay is currently in the process of replacing the Mantoux test in humans (Schiller et al., 2010) and is considered a good alternative for the skin test. Briefly, in humans the typical

cytokine profile indicates that at initial stages of infection *M. tb* specific immunity is predominantly Th1 mediated. Tumor necrosis factor (TNF-α) and IFN-γ are produced and activate macrophages to kill engulfed mycobacteria. In addition, killing of macrophages infected by mycobacteria, may be mediated by CD_4 cells, producing lymphotoxin and by perforin and granulysin produced by CD_8, γδ T-cell and CD_1 restricted T-cells (Kaufman, 2002). Activity of these cells is facilitated by IFN-γ and TNF-α. Currently, IFN-γ assays have been developed for humans (Taggart et al., 2004), cattle (Vordermeier et al., 2001 and Vordermeier et al., 2006), domestic cats (Rhodes et al., 2008), lions (Maas et al., 2012) and rhinoceros (Morar et al., 2007).

The present study aimed to develop a capture ELISA for the quantification of IFN-γ in Asian as wells as African *(Loxodonta africana)* elephants after stimulation with crude protein extracts of mycobacteria (PPD-B, PPD-A) and MTBC specific antigens (ESAT-6, CFP-10). Initial results confirmed detection of IFN-γ in concentration ranges comparable to those elicited by MTBC specific antigens in whole blood cultures of other species (Andersen et al., 2007, Morar et al., 2007 and Maas et al., 2012). Thus the IFN- γ assay an important tool for early detection of MTBC infection in many species, may be added to the diagnostic potential for tuberculosis in elephants.

Materials and Methods

1. Expression of eukaryotic recombinant elephant *IFN-γ* (*rEpIFN-γ*)

Eukaryotic recombinant elephant IFN- γ was produced by U-Protein Express BV, Utrecht, The Netherlands. The gene encoding *rEpIFN-γ* (Morar D, PhD Thesis University of Pretoria, 2009; Figure 1) was synthesized by GeneArt and cloned into a pUPE expression plasmid, that was transiently transfected into HEK293 EBNA cells as described by Durocher et al., 2002. Expression products were harvested from culture supernatants six days post-transfection.

(a)

GGCCTAACTCTCTCTGAAACAATGAATTTTACAAGTTATATCTTAGCTTTTCAGCTTTGC
ATCATTTTGGGTTCTTCTAGCTGCTACTGCCAGGCTACTTTTTTGAAAGAGATACAGAAC
CTAAAGGAATATCTTAATGCAACTGATTCAGATGTAGCGGATGGTGGGCCTCTTTTCATA
GATATTTTGAAGAACTGGAAAGAGGAGAGTGACAAAAAAATAATTCAGAGCCAGATCGTT
TCCTTTTACCTCAAAATCTTTGACAACTTGAAAGACAACCAGGTCATCCAAGAGAGCGTG
AAGACCCTTGAGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGAT
GACTTCCTAAAAGTGATGCAAACTCCGGTAAATGACCGGAACGTCCAGCGCAAAGCCATA
AGTGAGCTCTCCAAGGTGATGAATGACCTGTCACACAGATCTAACGGGGCAAACGAAAAA
GGAGACAGTATTCGTTTCGAGGCCGGAGAGCATCAGAATAATGG

(b)

MNFTSYILAFQLCIILGSSSCYCQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK
EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ
TPVNDRNVQRKAISELSKVMNDLSHRSNGAKRKRRQYSFRGRRASE

(c)

CAGGCTACTTTTTTGAAAGAGATACAGAAC
CTAAAGGAATATCTTAATGCAACTGATTCAGATGTAGCGGATGGTGGGCCTCTTTTCATA
GATATTTTGAAGAACTGGAAAGAGGAGAGTGACAAAAAAATAATTCAGAGCCAGATCGTT
TCCTTTTACCTCAAAATCTTTGACAACTTGAAAGACAACCAGGTCATCCAAGAGAGCGTG
AAGACCCTTGAGGAAGACCTCTTTGTTAAGTTCTTCAATAGCAGCTCCAGCAAACGGGAT
GACTTCCTAAAAGTGATGCAAACTCCGGTAAATGACCGGAACGTCCAGCGCAAAGCCATA
AGTGAGCTCTCCAAGGTGATGAATGACCTGTCACACAGATCTAACGGGGCAAACGAAAAA
GGAGACAGTATTCGTTTCGAGGCCGGAGAGCATCAGAATAA

(d)

GSQATFLKEIQNLKEYLNATDSDVADGGPLFIDILKNWK
EDSDKKIIQSQIVSFYLKIFDNLKDNQVIQESVKTLEEDLFVKFFNSSSSKRDDFLKVMQ
TPVNDRNVQRKAISELSKVMNDLSHRSSNGAKRKRRQYSFRGRRASE **AA**

Figure 1. Nucleotide and translated amino acid sequence of the synthetic gene for recombinant elephant IFN-γ (rEpIFN-γ) used in this study. (a) The real nucleotide sequence of the EpIFN-γ (Jasmin) gene. Selected primers used for the cloning are underlined (Morar, 2009). (b) The predicted protein sequence resulting from (a). (c) The synthetic nucleotide sequence that was ligated into pUPE expression vectors. (d) The predicted protein sequence resulting from (c).

2. Production of monoclonal antibodies

Immunization Two mice (BALB/c females; Charles River, Someren, Netherlands) were immunized intraperitoneally with 50 µg *rEpIFN-γ* in 50µl PBS mixed with 50µl Specol (Stimune® Prionics®, the Netherlands) on days 0, 21 and 42. On days 63 and 64 the immune response of the mice was boosted intravenously with 50 µg *rEpIFN-γ* in PBS.

Cell fusion and cloning. On day 67 spleen cells of the immunized mice were fused with mouse myeloma cells according to routine procedures. Sp2/0 using 40% polyethylene glycol 4000 (Art.9727 – Merck®). For two weeks hybridoma's were cultured in OptiMEM (Invitrogen®) medium supplemented with 10% Fetal Clone I (Hyclone®, USA),

hypoxanthine, aminopterin, thymidine and supernatants were screened by indirect ELISA (see below). Finally, selected hybridoma cultures were subcloned by a BD Influx Cell Sorter (BD Biosciences®, USA).

Isotyping and affinity purification. Monoclonal antibodies (MoAbs) were harvested and isotyped using the Beadlyte Mouse Immunoglobulin Isotyping Kit (Millipore®, The Netherlands) and the Luminex 100TM System according to the manufacturer's instructions.

MoAbs were affinity-purified, using GammaBindPlus Sepharose according to the manufacturer's instructions (GE Healthcare Europe GmbH). Subsequently, 1 mg of each MoAb was biotinylated with Biotin-ϵ-aminocaproic acid N-hydroxysuccinimide ester (biotin-X-NHS) according to the manufacturer's instructions (Roche® Diagnostics, The Netherlands).

Indirect ELISA. Hybridoma supernatants were screened for *rEpIFN-γ* specific antibodies by indirect ELISA. All incubations were done on a microtiterplate-shaker at 300 rpm at room temperature (RT). Ninety-six well ELISA plates (Costar®, Corning Incorporated, USA) were coated with *rEpIFN-γ* (5µg/ml, 50 µl/well) in phosphate buffered saline (PBS) for 1 hour, Antigen was discarded and the plates were blocked with 200 µl/well of block buffer (Roche® Diagnostics, The Netherlands) for 30 minutes. Block buffer was discarded and 50µl of hybridoma supernatants were added to the wells and incubated for 1 hour. Subsequently plates were washed 3 times with phosphate buffered saline containing 0.1% Tween 20 (PBST) and, polyclonal rabbit anti-mouse IgG-HRP 1:6000 (Southern Biotech® Alabama, USA), was added (50µl/well). After 1 hour incubation, plates were washed 4 times with PBST and once with tap water. Finally, 100µl/ well substrate 2,2'-azino-di (3-ethylbenzthiazoline-6-sulfonate) in a glycine/citric acid buffer (ABTS) (Roche®Diagnostics,

The Netherlands) was added. After 30 minutes of incubation, colour development was measured at 405 nm (Biorad microplate reader 550®, USA).

In addition, after purification and biotinylation of monoclonal antibodies, indirect ELISAs as described above were performed to select the MoAbs (biotinylated) with the highest affinity to *rEpIFN-γ*. For this, *rEpIFN-γ* was coated at 2 μg/ml (50 μl) and (biotinylated) MoAbs were added in 4-fold dilutions from 10 to 0.0024 μg/ml.

Selection of monoclonal antibodies for the IFNy capture ELISA. To select the optimal antibody tandem for the IFN-γ capture ELISA plates were coated with 50 μl (5 μg/ml) of capture monoclonal antibodies (non-biotinylated) per well. After incubation for 30 minutes and washing, *rEpIFN-γ* was added in 50 μl (1 μg/ml) per well, after subsequent incubation for 30 minutes and washing, biotinylated detection monoclonal antibodies were added in 50 μl (1 μg/ml) per well. After incubation for 2 hours and washing, the conjugate streptavidin-peroxidase (Biosource®) 1:2,000 was added. In subsequent assays to fine tune selection of antibody combinations *rEpIFN-γ* dilution series of 200-0.2 ng/ml were used in the same ELISA set up. The most sensitive combination was selected and used in the capture ELISA.

Western blot assay. The *rEpIFN-γ* was loaded onto precast Criterion gels (Biorad®) at concentrations of 11.66 μg/ml and 21μg/ml with a marker (precision plus protein dual colour, Biorad®) in one of the lanes. To determine which antibodies were binding linear epitopes, the recombinant *IFN-γ* was heated to 100℃ for 10 minutes in laemmli buffer. The samples were run at 100V for 1 hour and separated proteins were transferred to nitrocellulose at 0.8 mA/cm^2 for 45 minutes. After transfer, the blot was placed in a roller bottle in 10 ml 0.05% Tween20/PBS and incubated for 1 hour on a bottle roller at RT. After discarding 0.05% Tween20/PBS, the blots were cut into strips and incubated in 10 ml (0.1 μg/ml) of the purified monoclonal antibodies: 16F10C9 and 10F4G11 in PBS and incubated for 10

minutes, washed 3 times with distilled water and incubated with goat anti mouse alkaline-phosphatase (Southern biotech®) 1:1,000 for 10 minutes. The incubation was followed by three wash steps, using water and the detection was performed by adding nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt in 67% (DMSO) (v/v) (NBT/BCIP substrate) (Roche®) at RT for 60 minutes. To stop the reaction, the nitrocellulose was washed with running water and the membrane was dried.

3. Whole blood stimulation

First, to optimize the assay two elephants were selected; an Asian elephant from Artis Zoo, Amsterdam and one African elephant from Beekse Bergen Safari Park, both in The Netherlands, that tested negative both in bacterial culture as well as the *TB STAT PAK®* assay. Second, based on a variety of results of mycobacterial culture and serology (*TB STAT PAK*, Chembio®) four Asian elephants were selected from the population of the National Elephant Institute, Thailand for preliminary assessment of IFN-γ responses to tuberculosis specific antigens like ESAT6 and CFP10 to assess their potential for differentiation between infected and non infected elephants. Two of these elephants had never had contact with TB infected individuals and were found negative in the TB *STAT PAK®* assay for 2 years (non infected). One elephant, was suspected of TB because of a positive result in the *TB STAT PAK®* assay during more than 2 years, but its trunk wash culture was never found positive. The fourth elephant was shown to be infected with *M. tb* strain ATCC 27294 (modern type strain), in trunk wash culture (Angkawanish et al., 2012).

Both Asian and African elephant blood samples were collected in heparinized tubes (vaccutainer®) 20 ml, kept at 4℃ and the samples were processed within 6 hours. Whole blood was diluted 1:1 with complete medium (RPMI1640 + Glutamax containing 5%FCS,

50U/ml penicillin, 50U/ml streptomycin, 5,000 U heparin, 5x10^{-5} M 2-mercaptoethanol and L-glutamine (Gibco®) and incubated in 24-well tissue culture plates at 37℃, 5%CO_2 for 48 hours with mitogens used as positive control stimulants including pokeweed (PWM-lectin, Sigma®) 5 µg/ml and phorbol myristate acetate plus ionomycin (PMA/I, Sigma®, 100 ng/ml and 2 ug/ml) and the antigens avian and bovine tuberculin (PPDA and PPDB, synbiotics®) 25 U/ml, the hybrid of ESAT6 and CFP10 protein (E6/CFP10, Staten Serum Institute, DK) 1 µg/ml and ESAT6 (Staten Serum Institute, DK) 1 ug/ml. Culture medium was used as the negative control.

After incubation, blood samples were centrifuged at 3,200 RPM for 10 minutes and the supernatants were harvested and stored at -80℃ until tested in the optimized IFN- γ ELISA, described below.

4. Elephant *IFN-γ* capture ELISA, optimized protocol

ELISA plates (Greiner® Microlon, extra high binding 655061) were coated with 50 µl of 2 µg/ml capture antibody (MoAb 16F10C9) in PBS for 1 hour at RT. Plates were blocked with 1.3% casein in PBS (universal casein diluents, SDT®) for 1 hour at RT, emptied and washed 4 times with PBS/0.05% Tween-20. The supernatants of the whole blood cultures were diluted 1:1 with 1.3% casein buffer and added in triplicate. To produce a *rEpIFN-γ* standard curve a two-fold dilution series (500pg/ml – 0.5pg/ml in 1.3% casein buffer), was included in the assay. After 2 hours incubation at RT, plates were washed and 50µl/well biotinylated detection antibodies (MoAb 10F4G11) 1: 20,000 (0.025 µg/ml) diluted in 0.65% casein buffer, were added for 1 hour at RT. Plates were washed and streptavidin-peroxidase (SA-HRP80) diluted 1: 20,000 in 50 µl/well 0.5% casein buffer was added for 30 minutes at RT. Plates were washed, substrate 3,3',5,5'-Tetramethylbenzidine (TMB reagent) (SDT®, extra

sensitive) was added and the color reaction was stopped after 10 minutes using 1% HCl (1M; 50ul/well) and optical density (OD) was determined at 450 nm.

5. Data analysis

Responses measured by the IFN- γ ELISA were considered positive when higher than twice the average OD value of the negative (medium) control responses.

Results

The *IFN-γ* assay

The *rEpIFN-γ* produced in the eukaryotic cell line was used to immunize mice for production of MoAbs needed for the development of the capture ELISA. In addition it was used as the positive control in that assay. Hybridoma's identified as producers of IFN-*γ* specific antibodies in the indirect ELISA (n=11) were subcloned (Table 1). These antibodies, all identified as IgG1, kappa isotype, were subsequently purified. Finally 7 MoAbs were selected based on specific *rEpIFN- γ* binding and good growth capabilities of the corresponding hybridoma's to find the optimal combination for the capture ELISA. The combination of MoAb AE16F10C9 as capture antibody and MoAb AE10F4G11 as detection antibody showed the highest sensitivity (Figure 2). The Western blot assay confirmed the binding of *rEpIFN-γ* by those two antibodies (Figure 3). The typical *rEpIFN-γ* titration curve capture MoAb (AE16F10C9) and the detection MoAb (AE10F4G11) in the capture ELISA using optimal conditions as described above is shown in Figure 4. Detection levels ranged between 1 and 10,000 pg/ml of *rEpIFN- γ*.

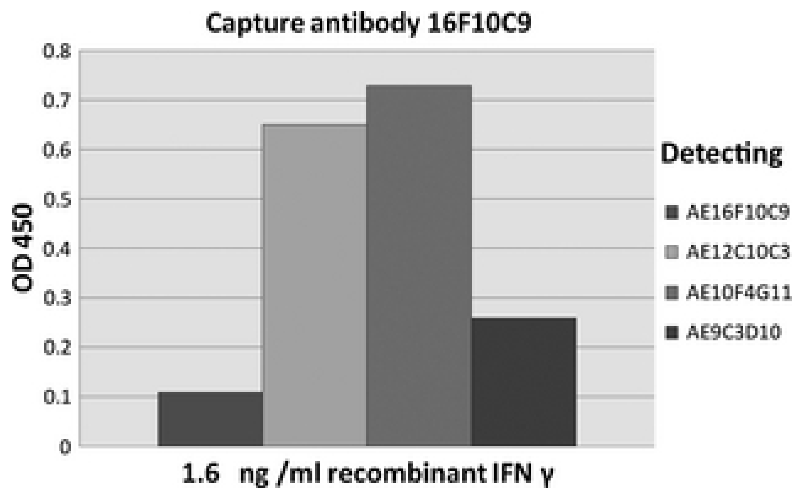

Figure 2. Combinations of capture and detection antibodies in the capture ELISA. Performance of capture antibody AE16F10C9 with four different detection antibodies is shown, at a concentration of 1.6 ng/ml recombinant IFN-γ. Capture antibody AE16F10C9 and detection antibody AE10F4G11 was the combination of choice in the optimized capture ELISA protocol.

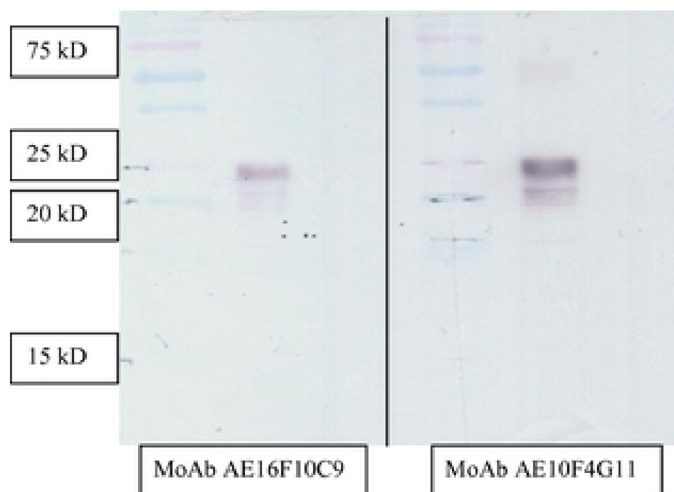

Figure 3. Monoclonal antibodies (MoAbs) detect recombinant elephant IFN-γ in Western blot. Capture (left) and detecting (right) MoAbs of choice recognize eukaryotic recombinant elephant IFN-γ (MW 25 kD) in Western blot.

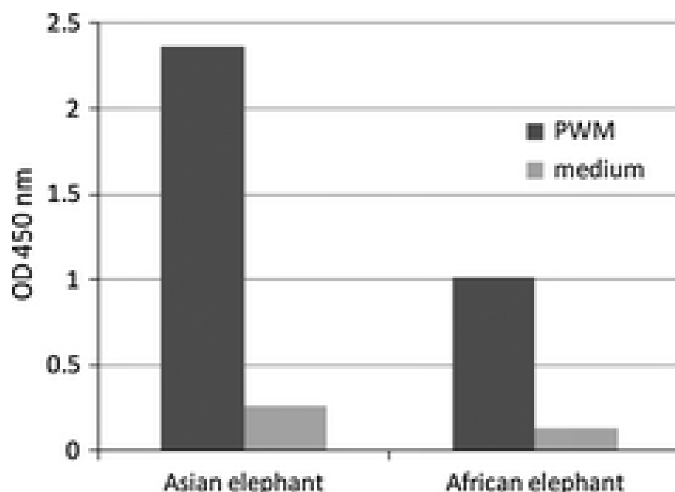

Figure 4. Titration curve of recombinant elephant IFN γ using the optimized capture ELISA. Eukaryotic recombinant elephant IFN γ was titrated in the optimized capture ELISA with capture MoAb (AE 16F10C9) and detection MoAb (AE 10F4G11). The lower detection limit was defined as 1 pg/ml.

Table 1. Monoclonal antibodies specific for elephant IFN-γ and their reactivity in indirect ELISA. Monoclonal antibodies (n = 11) specific for recombinant elephant IFN-γ and their relative affinities

1. MoAb, monoclonal antibody; IFN-γ, interferon gamma.
2. [a] Interaction with HIS-tag.

MoAb	**Reactivity**
AE16D7	+
AE11A7a	+
AE10F4	++
AE12C10	++
AE15A4	++
AE11D3	++
AE13B4	+
AE11G9a	++
AE16F11	++
AE9C3	++
AE16F10	+
Strong reactivity	++
Moderate reactivity	+

1. MoAb, monoclonal antibody; IFN-γ, interferon gamma.
2. [a] Interaction with HIS-tag.

Detection of native elephant *IFN- γ*

Assays using supernatants of whole blood cultures of the African and Asian elephants from MTBC free areas (NL) stimulated with PWM showed high OD with means of 1.00 and 2.36 respectively (Figure 5) as compared with the medium (negative control) stimulated cultures that showed mean OD's of 0.12 and 0.26. The assay showed to be applicable for both Asian and African elephants.

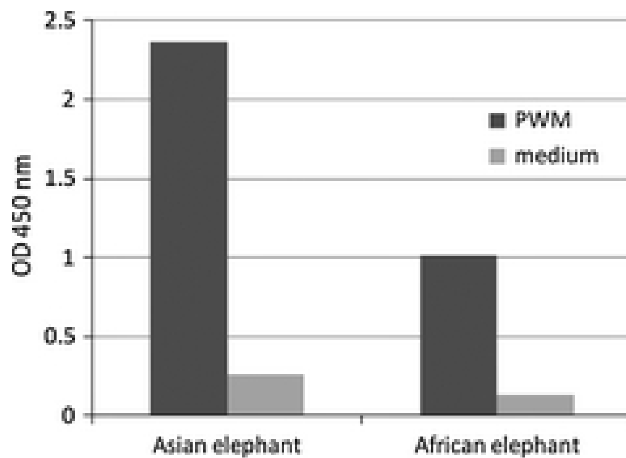

Figure 5. Recognition of native African and Asian IFN-γ in the capture ELISA. Whole-blood cultures (duplicates) of an Asian and African elephant were stimulated with mitogen in duplicate.

Table 2. Detection, of IFN-γ among four Asian elephants of different TB status. IFN-γ ELISA results (OD) in two non-infected, one TB-suspected and one *M. tb*-infected elephants after 24 h whole-blood stimulation (duplicates) with PWM, PMA/I as positive controls, and the MTBC-specific antigens ESAT6/CFP10 (EC) and PPDB. PPDA and medium were (negative) control stimulants

Stimulant	**Non-infected elephant 1**	**Non-infected elephant 2**	**TB-suspected elephant**	***M. tb*-infected elephant**
PWM	1.577 ± 0.24	1.058 ± 0.19	1.867 ± 0.19	2.569 ± 0.43
PMA/I	2.021 ± 0.02	1.976 ± 0.05	2.563 ± 0.12	2.572 ± 0.55
EC	0.103 ± 0.00	0.196 ± 0.03	0.129 ± 0.00	1.208 ± 0.02
PPDB	0.127 ± 0.03	0.164 ± 0.04	0.104 ± 0.03	0.530 ± 0.02
PPDA	0.132 ± 0.00	0.206 ± 0.01	0.123 ± 0.00	1.141 ± 0.10
Medium	0.080 ± 0.00	0.151 ± 0.03	0.062 ± 0.00	0.119 ± 0.00

1. IFN-γ, interferon gamma; *M. tb, Mycobacterium tuberculosis;* MTBC, *Mycobacterium tuberculosis* complex; PPD-B PPD-A, protein extracts of mycobacteria.

1.IFN-γ, interferon gamma; *M. tb, Mycobacterium tuberculosis;* MTBC, *Mycobacterium tuberculosis* complex; PPD-B PPD-A, protein extracts of mycobacteria.

In Table 2 results of IFN- γ assay are shown that confirm its ability to detect native elephant IFN-γ to a relevant level. The positive controls PWM and PMA/I reveal high OD values in all 4 Thai Asian elephant samples. Samples from non infected elephants were found negative upon stimulation with ESAT6/CFP10 PPDB and PPDA. The IFN-γ ELISA of supernatants of

whole blood culture of the elephant suspected of TB is negative (ESAT6/CFP10 borderline positive), whereas OD values after stimulation with ESAT6/CFP10 fusion protein, PPDB and PPDA are lower than the OD values of the non infected elephants. Finally, test results of the infected TB elephant were positive for all antigens ESAT6/CFP10 fusion protein, PPDB and PPDA with high OD450 values (1.208, 0.53 and 1.141, respectively).

Discussion

Availability of MoAbs specific for *rEpIFN- γ* and native elephant IFN- γ is an important pre-requisite for the development of an IFN- γ release assay for the diagnosis of tuberculosis in elephants. Antibodies produced showed cross-reactivity to both Asian and African elephant IFN- γ. The lower detection limit of the assay, 1 pg/ml *rEpIFN- γ,* seems adequate to assess responses elicited by MTBC specific antigens. In humans in highly endemic areas such as Ethiopia, ESAT-6 responses showed IFN-γ levels in the range 0-100 pg/ml in the lowest 30% of responders (Andersen et al., 2007). Similar levels were also observed in other species like cats (Rhodes et al., 2008).

In preliminary attempts to assess the assay in a TB setting whole blood samples of two non infected and one TB suspected elephant did not show responsiveness to ESAT6/CFP10, PPDB and PPDA, while those of the confirmed infected elephant showed IFN-γ production in response to these MTBC specific antigens as well as PPDA, the latter most likely due to cross-reactivity with environmental mycobacteria such as *M. avium* (Rhodes et al., 2008). The assay needs further optimization using pathogen specific antigens in addition to CFP10, ESAT6 and may have potential for differentiating between non infected and infected elephants (Vordermeier et al., 2001 and Andersen et al., 2007). In recent years, the IFN-γ assay has been complementing the tuberculin skin test in humans and cattle, facilitating early detection and follow up of disease progression. The *IFN-γ* assay is now established for both

Asian and African elephants, but needs further validation for its use in diagnosis which relies on its application in large populations of non infected, suspected and infected elephants.

Acknowledgements

We gratefully acknowledge Sittidet Mahasawangkul (the National Elephant Institute, Thailand), Bjarne Clausen (Danish Society Animal Welfare, Denmark), Konstantin Lyashchenko (Chembio Diagnostic, USA), Claus Aagaard, Peter Andersen (Staten Serum Institute Denmark) and Chartchote Thitaram (Chiangmai university, Thailand) for providing valuable information. This work was supported by the EU-Asia Link project**,** TH/Asia-Link/012(141-055).

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