Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) Interferon-gamma (IFN-γ) and the production of rhinoceros IFN-γ specific antibodies

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Introduction

Mycobacterium bovis **has been found to have an exceptionally wide host range which includes domesticated ruminants and captive and free-ranging wildlife. . Bovine tuberculosis has to date not been diagnosed in pachyderms in South Africa. Potential spillover into the rhinoceros population can occur, and it is of utmost importance to be able to provide an additional guarantee on the Tb free status of these animals and to provide conservation bodies with an early warning system should bovine Tb enter the rhino population. Described here are the first steps in developing an IFN-γ based ELISA for the detection of *M. bovis* infection in white rhinoceros *(Ceratotherium simum)* and we also report the first evaluation of the specificity of the test in known Tb free rhinoceros. The set up of the test included the cloning, sequencing and the expression of the white rhinoceros IFN-γ (RhIFN-γ) gene and the production of the RhIFN-γ specific monoclonal and polyclonal antibodies. Thus, the IFN-γ produced in vitro by antigen stimulation of sensitised T-lymphocytes can be measured to serve as a sensitive and specific indicator of *M. bovis* exposure.**

Materials and Methods

Cloning, sequencing and expression of the white rhinoceros (Ceratotherium simum) IFN-γ gene

Peripheral mononuclear cells (PBMC) of white rhinoceros were stimulated with mitogen Concanavalin A (Con A). After 20 hrs the cells were harvested and total RNA was isolated and was used to produce cDNA. This cDNA was used as a template in amplification reactions with IFN-γ primers which were selected specific for regions that showed the highest degree of conservation between mammals. The PCR product was cloned and the DNA sequence was determined. Once the complete sequence of the IFN-γ gene was known, the PCR product of the complete coding sequence was cloned using the Gateway Cloning Technique (Invitrogen) and the protein was expressed and purified.

White rhinoceros IFN-γ specific poly- and monoclonal antibodies

Balb/C mice were immunized with the purified recombinant IFN-γ. Hybridomas were selected that produced antibodies specific for the purified IFN-γ. Sets of monoclonal antibodies were selected and used in an ELISA protocol and assay conditions were optimized to be used in the IFN-γ assay. For the production of polyclonal antibodies, chickens were immunised intramuscularly and boosted on a regular basis to maintain antibody titres. Antibodies were purified from the egg yolk by the "water dilution method" followed by ammonium sulphate precipitation according to the procedure described by Hansen *et al.,* 1998. After extensive dialysis against PBS, poly- and monoclonal antibodies were sterile filtered using a 0.2 M filter.

Native IFN-γ

Blood was collected in EDTA Vacutainer tubes from three Tb free white rhinoceros. PBMC were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Amersham 17-1440-02). To induce IFN-γ production, purified mononuclear cells ($1X10^6$ cells/ml) were stimulated with 10 μg/ml Con A (Sigma, C2010-100mg) at 37°C in a 5% CO_2 incubator. PBMC were also stimulated with Bovine and Avian purified protein derivatives (PPD). A control sample was included consisting of PBMC cultured without mitogen. After 18 to 24 hours incubation, cell cultures were collected and centrifuged at 3200 rpm for 10 mins and the supernatant harvested. Production of IFN-γ was analysed in the capture ELISA described below.

Prototype capture ELISA for detection of native white rhinoceros IFN-γ

ELISA plates (Nunc, C96 446140) were coated with monoclonal antibody (mAb) 1H11 and incubated overnight at 4°C. Wells were blocked with block buffer (2% fat free milk powder in 1 X PBS) and incubated at 37°C for 1 hr. The plates were washed with wash buffer (H_2O/0.1% Tween 20) five times. As a positive control recombinant white rhinoceros IFN-γ was diluted in PBS and tested in duplicate. Undiluted supernatants collected from overnight stimulated PBMC were added to the remainder of the wells. After the incubation the wells were washed five times with wash buffer and incubated with polyclonal antibodies to white rhinoceros IFN-γ per well. After 1 hr the plates were washed five times with wash buffer and rabbit polyclonal to chicken IgY H&L (HRP) (Abcam, ab6753) antibody was added (1:3000 dilution). The wash step with wash buffer was repeated and the addition of o-phenylenediamine (OPD) (Sigma, P3804) substrate followed. The reaction was stopped after 20 mins with 2N H_2SO_4 and the OD was read 10 mins later at 492nm.

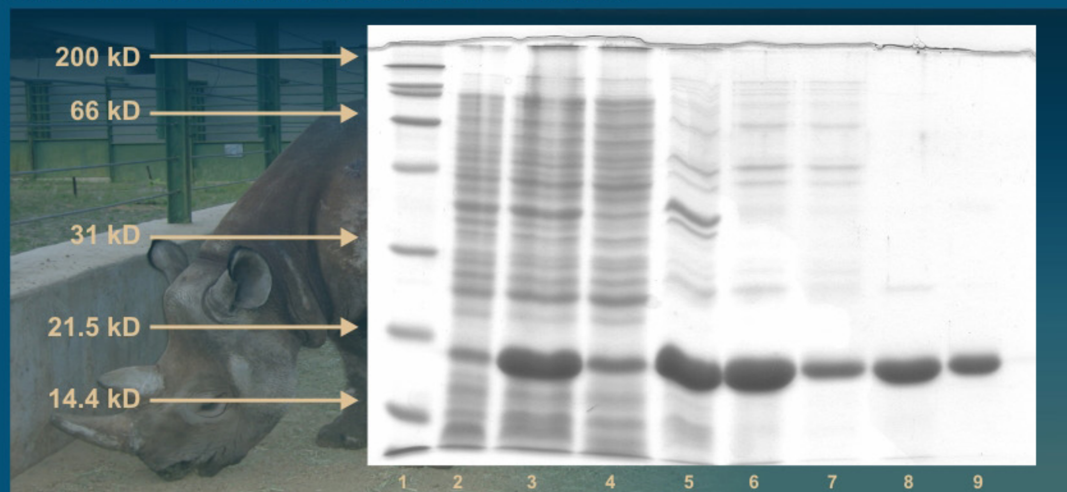
Results

Cloning and sequencing of the white rhinoceros (Ceratotherium simum) IFN-γ gene

The coding part of the white rhinoceros IFN-γ gene is 501 nucleotides long and encodes a protein with a predicted molecular weight (MW) of 19.4 kDa. Blast searches of the nucleotide sequences and the predicted aa sequences of RhIFN-γ demonstrated the highest homology with equine IFN-γ, that is 90% identity on the nucleotide as well as on the aa level

Expression and purification of recombinant white rhinoceros IFN-γ (rRhIFN-γ)

The major part of the expressed rRhIFN-γ was present in the insoluble fraction as inclusion bodies (Fig. 1, Lane 5). After solubilisation of the inclusion bodies in 8 M Urea (Fig. 1, Lane 6), the majority of the hexahistidine-tagged rRhIFN-γ bound to a column with immobilized $Ni2^+$ (Fig. 1, Lane 8) and a minor part failed to bind and showed up in the flow through fraction (Fig. 1, Lane 7). After washing, the bound protein was refolded on the column. After elution and dialysis the refolded IFN-γ had a purity of at least 95% (Fig. 1, Lane 9).



Lane 1. Broad Range Mw Marker, Lane 2. Total bacterial lysate (uninduced), Lane 3. Total bacterial lysate (IPTG-induced), Lane 4. Soluble fraction, Lane 5. Insoluble fraction (inclusion bodies), Lane 6. Solved inclusion bodies (10 M urea), Lane 7. Flow-through Ni2+ column, Lane 8. Protein bound on column, Lane 9. Eluted protein

Figure 1: SDS-PAGE gel showing the purification of recombinant IFN-γ

Prototype sandwich ELISA for the detection of white rhinoceros IFN-γ

Recombinant white Rhinoceros IFN-γ could be detected using the preliminary sandwich ELISA, using mAb 1H11 as a capture antibody in combination with polyclonal anti-IFN-γ IgY as a detecting antibody. This sandwich ELISA was also able to detect native white rhinoceros IFN-γ (Fig. 2), as was demonstrated by the strong signal obtained with supernatants of PBMC of three white rhinoceros that had been stimulated with Con A to induce the expression of this cytokine.

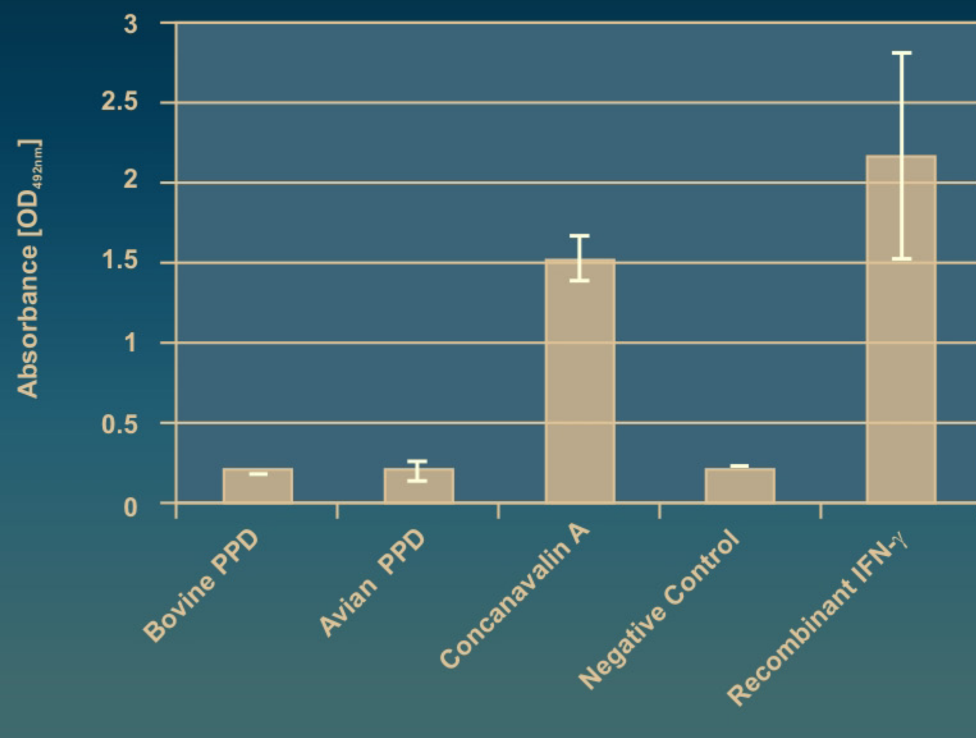


Figure 2: Detection of white rhinoceros IFN-γ in PBMC stimulated with Bovine PPD, Avian PPD and Concanavalin A using the IFN-γ Sandwich ELISA. Results are expressed as the mean value from three rhinoceros. ⊥ indicated the standard deviations.

Discussion and Conclusion

The white rhinoceros IFN-γ gene was successfully cloned, sequenced and expressed. Monoclonal antibodies specific for rRhIFN-γ were also produced. A prototype sandwich ELISA could be developed, using antibody 1H11 in combination with polyclonal chicken antibodies that are able to detect both recombinant and native white rhinoceros IFN-γ.

Tests have only been performed in white rhinoceros and have yet to be performed in black rhinoceros. A first confirmation of the specificity of the test was done using Tb free white rhinoceros. No IFN-γ was detected after PPD stimulation although a positive signal was detected after Con A stimulation of PBMC. In conclusion, although optimization of the ELISA with respect to its evaluation for Tb *(M.bovis / M. tuberculosis)* infected rhinoceros need to be conducted, the sandwich ELISA as designed demonstrated to be a promising approach towards diagnosis of Tb *(M.bovis / M. tuberculosis)* infection in the white rhinoceros.

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References

Buddle *et al.*, Veterinary Immunology and Immunopathology., 2000, 74:1-16; Hansen *et al.*, Journal of Immunological Methods., 1998, 215:1-7; Michel *et al.*, Veterinary Microbiology 2006, 112:91-100;