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CLONING, SEQUENCING, AND EXPRESSION OF INTERFERON-γ FROM ELK IN NORTH AMERICA

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ABSTRACT: Eradication of *Mycobacterium bovis* relies on accurate detection of infected animals, including potential domestic and wildlife reservoirs. Available diagnostic tests lack the sensitivity and specificity necessary for accurate detection, particularly in infected wildlife populations. Recently, an in vitro diagnostic test for cattle which measures plasma interferon-gamma (IFN-γ) levels in blood following in vitro incubation with *M. bovis* purified protein derivative has been enveloped. This test appears to have increased sensitivity over traditional testing. Unfortunately, it does not detect IFN-γ from Cervidae. To begin to address this problem, the IFN-γ gene from elk (*Cervus elaphus*) was cloned, sequenced, expressed, and characterized. cDNA was cloned from mitogen stimulated peripheral blood mononuclear cells. The predicted amino acid (aa) sequence was compared to known sequences from cattle, sheep, goats, red deer (*Cervus elaphus*), humans, and mice. Biological activity of the recombinant elk IFN-γ (rElkIFN-γ) was confirmed in a vesicular stomatitis virus cytopathic effect reduction assay. Production of monoclonal antibodies to IFN-γ epitopes conserved between ruminant species could provide an important tool for the development of reliable, practical diagnostic assays for detection of a delayed type hypersensitivity response to a variety of persistent infectious agents in ruminants, including *M. bovis* and *Brucella abortus.* Moreover, development of these reagents will aid investigators in studies to explore immunological responses of elk that are associated with resistance to infectious diseases.

Key words: Bovine tuberculosis, Cervidae, *Cervus elaphus,* cytokine, diagnostic assay, elk, IFN-γ, *Mycobacterium bovis.*

INTRODUCTION

Eradication of *Mycobacterium bovis,* the causative agent of bovine tuberculosis, requires accurate detection of infected animals, including reservoirs of tuberculosis among domestic ruminants and wildlife. Recent cases of *M. bovis* infection in captive (Thoen et al., 1992; Nation et al., 1999) and free-ranging elk (*Cervus elaphus*) (Hunter, 1996) and in free-ranging white-tailed deer (*Odocoileus virginianus*) (Schmitt et al., 1997) emphasize the importance of Cervidae as potential reservoirs of tuberculosis among North American wildlife populations. Intradermal tuberculin testing with purified protein derivative (PPD), the standard method of tuberculosis detection, has been problematic in cervids due to varied sensitivity and specificity (Griffen and Buchan, 1994). Moreover, intradermal testing in Cervidae requires handling animals twice. This makes testing difficult in free-ranging elk or deer (Griffen et al., 1994). Several enzyme linked immunosorbent assay (ELISA)-based diagnostic tests have been developed to measure antibody responses to a variety of mycobacterial antigens (Sugden et al., 1997). Unfortunately, antibody levels tend to be low in subclinically infected animals and are therefore most effective in identifying animals with disseminated disease (Palmer et al., 2000). An alternative combination of blood tests for tuberculosis (BTB) has been developed and is currently in use in New Zealand. These tests measure antibody, inflammation and lymphocyte proliferation in response to PPD from different mycobacteria (Griffen et al., 1994). However, recent studies in Canada showed the BTB test had a sensitivity of only 76% and specificity of 77% (Hutchings and Wilson, 1995). Additionally, because the proliferation test customarily measures total tritiated thymidine

incorporation after culturing lymphocytes, it requires special handling procedures by skilled technicians trained in handling radionuclides.

Interferon-gamma (IFN-γ), a cytokine produced by sensitized T lymphocytes, is known to be important in the immune response to many intracellular bacteria via its effect on macrophage activation and induction of class I and class II MHC (Russell, 1995). The delayed type hypersensitivity (DTH) reaction, an immune response to certain antigens, is mediated by a population of sensitized T lymphocytes that interacts with antigen presented by macrophages in association with MHC class II (Barnes et al., 1993; Monaghan et al., 1994). This interaction results in the release of IL-2 and IFN-γ and the subsequent tissue damage, swelling, and induration characteristic of a positive reaction (Monaghan et al., 1994; Oliveira et al., 1996).

The response to PPD from mycobacteria and *Brucella* spp., measured by certain diagnostic tests for detection of tuberculosis and brucellosis respectively, is a DTH reaction (Wood et al., 1992; Monaghan et al., 1994; Weynants et al., 1995). The observation that T cells previously sensitized to these bacterial antigens release relatively large amounts of IFN-γ when exposed to PPD has led to the recent development of a new in vitro diagnostic test for *M. bovis.* Rothel et al. (1990, 1992) describe a test for *M. bovis* detection in cattle that compares levels of IFN-γ from heparinized whole blood incubated with *M. bovis* versus *Mycobacterium avium* PPD. Interferon-γ levels following incubation are measured by an ELISA, utilizing a monoclonal antibody (mAb) developed against bovine IFN-γ. The bovine IFN-γ assay appears to be more sensitive than skin testing, requires handling animals only once, and bypasses potential problems with hyposensitization (Wood and Rothel, 1994). However, while the mAb recognizes IFN-γ from cattle, sheep, Australian buffalo (*Bubalus bubalis*) and goats, it does not detect IFN-γ from cervids (Rothel et al., 1990).

As a first step in development of an IFN-γ-based ELISA for *M. bovis* detection in Cervidae, this paper describes the cloning, sequencing, characterization and expression of recombinant IFN-γ from elk. We hypothesize that IFN-γ, produced in vitro by antigen stimulation of sensitized T lymphocytes, can be measured in cervid whole blood as a sensitive and specific indicator of *M. bovis* exposure. Successful development of this assay could yield a vital tool for detecting, controlling and eradicating bovine tuberculosis within domestic and free-ranging cervid populations and minimizing risk of transmission to cattle. An assay that measures IFN-γ expression after incubation of sensitized T cells with appropriate antigen could be used to detect other disease, such as brucellosis, as well (Weynants et al., 1995). Moreover, the IFN-γ sequence could provide an important tool for investigating immune responses of elk to infectious diseases.

MATERIALS AND METHODS

Lymphocyte purification

To obtain lymphocytes, blood from captive adult elk was collected by jugular venipuncture (four parts whole blood to one part acid citrate dextrose [2.45% dextrose, 2.2% sodium citrate, 0.73% citric acid, pH 7.2]). Lymphocytes were purified by differential centrifugation through Accu-Paque (sp. gr. 1.086) (Accurate Chemical and Scientific Corp., Westbury, New York, USA). Mononuclear cells at the interface were removed, washed three times and resuspended in complete RPMI-1640 culture medium (Gibco BRL, Gaithersburg, Maryland, USA), supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum. Thirty-two ml of elk blood routinely yielded approximately 4×10^7 viable lymphocytes.

Cloning, sequencing and characterization of the IFN-γ gene

To induce IFN-γ production, purified lymphocytes (5×10^6/ml) were stimulated with 2.5 μg/ml pokeweed mitogen and 5 μg/ml concanavalin A (ConA) (Sigma Chemical Co., St. Louis, Missouri, USA) for 18–24 hr at 37 C in a humidified 5% CO_2 incubator. Total RNA was purified from stimulated lymphocytes using

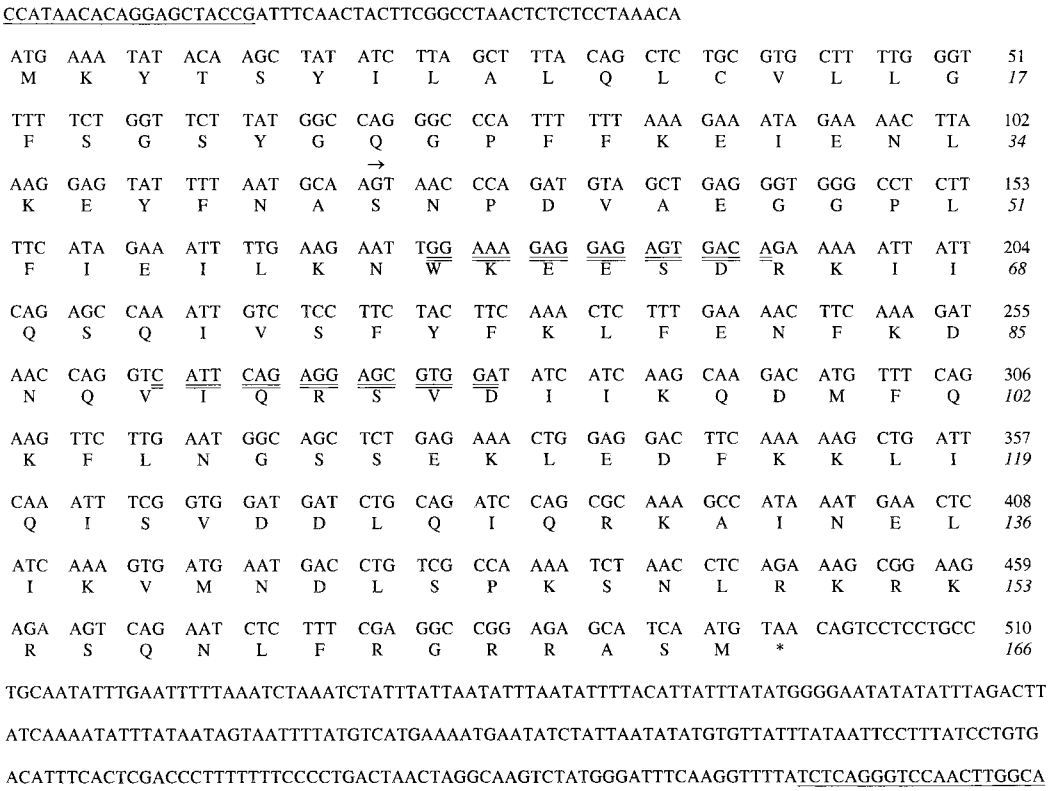

FIGURE 1. Nucleotide sequence and predicted amino acid sequence of elk IFN-γ. Single underlined regions denote oligonucleotide primers used for RT-PCR. Double underlined regions show interior primers used for final sequencing. Arrow below Gln-24 indicates hypothesized amino terminus of naturally secreted elk IFN-γ.

Trizol reagent (Life Technologies Inc., Rockville, Maryland, USA) and 1 μg of total RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) with MuLV reverse transcriptase and oligo $d(T)_{16}$ poly T tail primers, using the GeneAmp RNA PCR Kit (Perkin-Elmer, Foster City California, USA). Following reverse transcription, elk *IFN-γ* cDNA was synthesized by PCR using *IFN-γ*-specific primers from non-translated regions flanking the 5′ (primer sequence: 5′-CCA TAA CAC AGG AGC TAC CG-3′) and 3′ (primer sequence: 5′-TGC CAA GTT GGA CCC TGA GA-3′) ends of ovine *IFN-γ* gene (McInnes et al., 1990). Polymerase chain reaction was carried out for 35 cycles (one cycle consists of: 94 C 30 sec; 50 C 30 sec; 70 C 30 sec) in a 20 μl volume using a GeneAmp PCR System 2400 thermocycler. To facilitate sequencing, PCR products were initially cloned into pCR II cloning vector using the TA cloning kit (Invitrogen, San Diego, California, USA). Recombinant plasmids were doubly screened for ampicillin resistance. Recombinant plasmids were purified on columns using the Wizard DNA Clean-Up System (Promega, Madison, Wisconsin, USA) followed by agarose gel electrophoresis to verify recombinant plasmid size.

Several inserts were sequenced bidirectionally using the dideoxynucleotide chain termination method. Following initial sequencing, interior primers were constructed to complete sequencing of elk *IFN-γ* cDNA. Primers were 5′-GGA AAG AGG AGA GTG ACA G-3′ corresponding to the 5′ strand at base pair (bp) 176–194 and 5′-TCC ACG CTC CTC TGA ATG-3′ complementary to the 3′ strand at bp 264–281. To facilitate recombinant protein expression, selected portions of the recombinant pCR II plasmids were directionally subcloned into the 5′ *Nde*I and 3′ *BamH*I restriction enzyme sites of the pET 19b cloning vector (Novagen, Madison, Wisconsin, USA). Two cloning vectors were produced. One contained the entire *IFN-γ* cDNA sequence of 166 codons, and the second vector contained 143 codons, lacking the putative signal sequence (Cerretti et al., 1986). Recombinant pET plasmids were isolat-

```
                1                                                   50
elk             MKYTSYILAL QLCVLLGFSG SYGQGPFFKE IENLKEYFNA SNPDVAEGGP
red deer        ---------- ---------- ---------- ---------- ----------
ovine           -----SF--- L--------- ---------- ---------- ------K---
caprine         -----SF--- L-S------- ---------- ---------- ------K---
bovine          ------F--- L--G------ -----Q--R- ---------- -S----K---
human           ---------F ---IV--SL- C-C-D-YV-- A----K---- GHS---DN-T
murine          -NA-HC---- --.F-MAV-- C-CH-TVIES L-S-NN---S -GI--E-KS.

                51                                                  100
elk             LFIEILKNWK EESDRKIIQS QIVSFYFKLF ENFKDNQVIQ RSVDIIKQDM
red deer        ---------- ---------- ---------- ---------- ----------
ovine           --S------- ----K----- ---------- --L------- --M-------
caprine         --S------- ----K----- ---------- --L------- --M-------
bovine          --S------- D---K----- ---------- --L------- --M-------
human           --LG------ -------M-- ---------- K----D-S-- K--ET--E--
murine          --LD-WR--Q KDG-M--L-- --I---LR-- -VL----A-S NNISV-ESHL

                101                                                 150
elk             FQKFLNGSSE KLEDFKKLIQ ISVDDLQIQR KAINELIKVM NDLSPKSNLR
red deer        ---------- ---------- ---------- ---------- ----------
ovine           ---------- ------R--- -P-------- ---------- ----------
caprine         ---------- ---------- -P-------- ---------- ----------
bovine          ---------- ---------- -P-------- ---------- ----------
human           NV--F-SNKK -RD--E--TN Y--T--NV-- ---H---Q-- AE---AAKTG
murine          ITT-FSN-KA -KDA-MSIAK FE-NNP-V-- Q-F----R-V HQ-L-E-S--

                151            166
elk             KRKRSQNLFR GRRASM
red deer        ---------- -----T
ovine           ---------- ------
caprine         ---------- ------
bovine          ---------- -----T
human           ------M--- -----Q
murine          -----RC... ......
```

FIGURE 2. Alignment of elk, red deer (GenBank accession #L07502), ovine (GenBank accession #X52640) (McInnes et al., 1990), caprine (GenBank accession #U34232) (Beyer et al., 1998), bovine (GenBank accession #M29867) (Cerretti et al., 1986), human (GenBank accession #J00219) (Gray and Goeddel, 1982), and murine (GenBank accession #K00083 (Gray and Goeddel, 1983) IFN-γ amino acid sequences. Dashes indicate positions of homology relative to the elk IFN-γ sequence. Dots indicate no corresponding aa.

ed from transformed *Escherichia coli* XL1-Blue (Stratagene, La Jolla, California, USA) after single selection for ampicillin resistance. Randomly selected colonies were screened for *IFN-γ* cDNA inserts by *Pst*I restriction enzyme digestion, which yielded a linearized fragment of approximately 1,180 or 1,110 bp distinct from the linearized plasmid.

Digests were analyzed by agarose gel electrophoresis and clones sequenced by dideoxynucleotide chain termination to verify correct insertion and sequence fidelity. The Wisconsin Sequence Analysis Package (BLAST program) (Devereux et al., 1984) was then used to compare the *IFN-γ* cDNA sequence from elk to existing *IFN-γ* cDNA sequences in red deer, sheep, goats, cattle, humans, and mice (obtained from the GenBank database maintained at National Center for Biotechnology Information in Bethesda, Maryland).

Recombinant gene expression

Recombinant pET plasmids containing the 166- and 143-codon versions of recombinant elk IFN-γ (rElkIFN-γ) were used to transform *E. coli* BL21 (DE3) (Novagen). Single ampicillin resistant colonies were incubated in 50 ml LB-ampicillin with shaking at 37 C until OD_{600} reached 0.5–0.7 (4–5 hr). Expression was in-

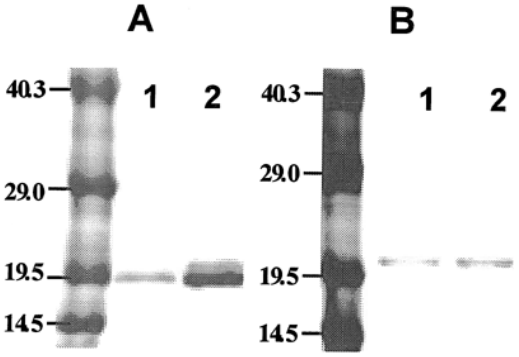

FIGURE 3. Coomassie blue-stained SDS-PAGE of purified (A) 143- and (B) 166-amino acid rElkIFN-γ protein. Both recombinant proteins include a plasmid-encoded peptide of about 2.7 kDa. Lanes 1 and 2 represent different elution fractions from nickel chelation columns. (A) Expressed protein for the 143 aa sequence yielded a mw band approximately 19.7 kDa, as predicted for His-IFN-γ lacking a signal peptide. (B) Expressed protein for the 166 aa sequence yielded a mw band of approximately 22.1 kDa, as predicted for His-IFN-γ with a signal peptide leader sequence.

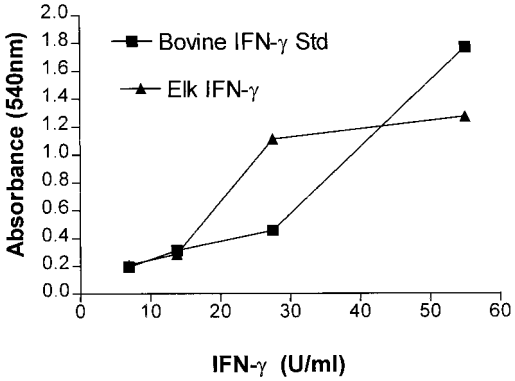

FIGURE 4. Inhibition of VSV plaque formation by rElkIFN-γ. Confluent MDBK cells were treated with serial dilutions of either rElkIFN-γ or recombinant bovine IFN-γ, which served as a standard. Treatments were performed in triplicate. Cells were then infected with virus for 24 hr and the cell viability was measured colorimetrically. Absorbance was measured at 540 nm on a Titertek Multiscan MCC/340 microtiter plate reader.

duced with 1 mM IPTG and incubation continued for 3 hr after which cells were placed on ice and harvested by centrifugation at 4,000 × *g* for 10 min. Cells were frozen at −86 C until ready for processing. Cell pellets were thawed on ice, and lysates were prepared by sonication. To purify the recombinant protein, a rapid affinity technique (Novagen) which employs a nickel-charged resin that binds a series of plasmid-encoded histidine residues at the amino terminus was used. The purified His-IFN-γ was eluted with 20 mM imidazole in 1 to 3 ml fractions. Purification was carried out under denaturing conditions using 6 M urea. The resulting recombinant protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight (mw) bands of approximately 22.1 and 19.7 kDa which included a plasmid-encoded peptide of about 2.7 kDa. The 19.7 kDa protein, which lacked the signal peptide, was dialyzed into 1× phosphate buffered saline (PBS) and ultrapure water and stored at −86 C.

Interferon-gamma bioassay

The biological activity of the rElkIFN-γ without the leader sequence was determined using an assay based on the reduction of vesicular stomatitis virus (VSV) cytopathic effect in Madin-Darby bovine kidney (MDBK) cells (Armstrong, 1981; Lewis, 1987). Madin-Darby bovine kidney cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum, L-glutamine (2 mM), Hepes buffer (10 mM) and gentamicin (50 μg/ml), were seeded into a 96-well, flat-bottomed microtiter plate at a density of 3×10^4 cells per well. The cells were allowed to adhere for 1–4 hr at 37 C in 5% CO_2. Confluent MDBK cells were treated with serial dilutions of either rElkIFN-γ or recombinant bovine IFN-γ as a reference standard for 24 hr at 37 C. The cells were then exposed to VSV for 24 hr and the cell viability was measured by reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co.) for 4 hr at 37 C in 5% CO_2. The reduced dye was extracted with 0.04 N HCl in isopropanol at 37 C for 16 to 20 hr. Absorbance was measured at 540 nm on a Titertek Multiscan MCC/340 microtiter plate reader. Triplicate wells of transfection medium alone added to MDBK cells and incubated in the presence or absence of VSV were used for controls.

RESULTS

Characterization of recombinant clones

Two clones of recombinant elk *IFN-γ* cDNA were produced. One clone contained an open reading frame (ORF) of 498 bp and encoded a polypeptide of 166 aa with a predicted mw of 19,445 Da (Fig. 1). The second clone contained an ORF of 429 bp that encodes a polypeptide of 143

aa with a predicted mw of 16,935 Da. The latter clone lacked the signal peptide, simulating naturally secreted elk IFN-γ. Like bovine IFN-γ, the amino terminus of elk IFN-γ is unknown but is assumed to be Gln-24 as in human IFN-γ (Rinderknecht et al., 1984). This leaves the first 23 aa with many features of an expected signal sequence for a secreted protein (Cerretti et al., 1986). Both recombinant elk *IFN-γ* clones contained predicted N-glycosylation sites at aa residues 39 and 106.

Homology to other IFN-γ

The predicted aa sequence of rElkIFN-γ was 92% homologous to bovine IFN-γ (Cerretti et al., 1986), 94% homologous to caprine (Beyer et al., 1998) and ovine IFN-γ (McInnes et al., 1990), and 99% homologous to red deer IFN-γ (GenBank accession #L07502). Homology to non-ruminant species was considerably less, with 67% homology to human IFN-γ (Gray and Goeddel, 1982) and 45% homology to murine IFN-γ (Gray and Goeddel, 1983) (Fig. 2).

Recombinant IFN-γ of all four ruminant species contained 166 aa residues. In red deer IFN-γ, a single nucleotide substitution (C for T) at bp 497 produced a Thr instead of a Met at the carboxy terminus. Cerretti et al. (1986) noted the same substitution in two otherwise identical clones of bovine IFN-γ but were unsure whether this reflects an allelic difference or a reverse transcriptase error in one of the cDNA constructs. Close agreement between IFN-γ sequences of elk and red deer should be expected, as these animals are classified as conspecific though native to different continents (Haigh and Hudson, 1993).

Protein expression and biological activity

Expressed protein yielded mw bands of approximately 19.7 and 22.1 kDa as predicted for His-IFN-γ polypeptides of 143 (Fig. 3A) and 166 aa (Fig. 3B). The smaller of the two proteins was much more concentrated, possibly because it lacked a signal peptide, causing more of the product to be retained by cells rather than being secreted into the culture medium. The biological activity of the rElkIFN-γ was confirmed with a VSV cytopathic effect reduction assay (Fig. 4).

DISCUSSION

The *IFN-γ* gene from elk was cloned, sequenced, expressed and characterized. In comparison with IFN-γ sequences from other ruminant species, elk IFN-γ had individual aa differences throughout the sequence. These differences likely account for the lack of reactivity of IFN-γ from Cervidae with the commercially available mAb against bovine IFN-γ. However, areas of homology could be used for peptide synthesis to produce mAb that would be cross-reactive with IFN-γ from both domestic and wild ruminants, allowing for the future development of a solid-phase ELISA that will detect IFN-γ from sensitized cervids as well as cattle.

Currently, reliance of the IFN-γ assay (as well as the intradermal and lymphocyte proliferation tests) on immune response to PPD tuberculins remains a major limitation. PPD is composed of a partially purified mixture of proteins, lipids, and sugars derived from whole organisms (Monaghan et al., 1994). Because many mycobacterial species are closely related, particularly in regards to their cell wall components, a number of the antigens that make up PPD are shared among several mycobacterial species. Thus, false positive reactions can occur in either test as a result of sensitization due to prior infection with other mycobacteria as well as related agents such as *Nocardia* sp. (Monaghan et al., 1994). In conjunction with the development of reagents to detect IFN-γ, our laboratory is currently working to identify *M. bovis*-specific, conserved T cell epitopes of antigens that specifically and sensitively stimulate a T cell response in *M. bovis*-infected lymphocytes. The utilization of more defined antigens that are both specific for *M. bovis* and can identify all

M. bovis isolates would overcome many of the problems associated with PPD-based antigen testing.

Interferon-γ is known to have an important role in the development of protective immune responses to many infectious diseases (Shalaby et al., 1984). Reagents that can detect and quantify elk IFN-γ could provide important tools for elucidating immune responses to different pathogens and characterizing the immunological responses associated with resistance to infectious diseases in elk. Moreover, the development of an IFN-γ-based ELISA could yield a vital tool for detecting, controlling and eradicating bovine tuberculosis, as well as other diseases such as brucellosis, within domestic and free-ranging cervid populations, thereby minimizing risk of disease transmission to cattle.

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