CONSTRUCTION AND OPTIMIZATION OF AN INTERFERON GAMMA DIPSTICK ASSAY FOR THE DETECTION OF ANTIGEN-SPECIFIC CELL MEDIATED IMMUNE RESPONSES

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Mara Elton

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Research Faculty Advisor: Jerrie Gavalchin

ABSTRACT

Since 1957, when interferons were discovered, Interferon gamma (IFN-γ) has played a crucial role as a diagnostic tool for both humans and animals. Because IFN-γ is an antigen-induced protein produced by blood lymphocytes, namely CD4+ T-cells, it is considered the first and foremost form of defense that the host has against infections. Two pathogens that induce a cell mediated immune response (CMI) are *Mycobacterium bovis* (*M. bovis),* which causes tuberculosis (TB), and *Mycobacterium avium paratuberculosis* (*Map*), which is the causative agent for Johne's disease. Both diseases pose serious health and economic problems for livestock production, particularly cattle. Clinical signs of these diseases are not apparent during the early stages of infection or in latently-infected animals. Nor are the levels of the mycobacterium high enough to detect in early disease. However, since exposure to tuberculin or Johnin antigen by skin testing induces a pathogen-specific CMI response with increased IFN-γ, it has been suggested that this assay, in combination, might be useful in the diagnosis of TB or Johne's. However, while IFN-γ assays are available, they have qualities that prohibit their routine use in the field: they are species specific, expensive to perform, require sophisticated laboratory equipment and trained personnel, and they are not quantitative. In this study, we report the construction and optimization of a lateral flow assay for determining levels of IFN-γ. This assay is also robust, easy-to-use and requires no sophisticated equipment, all qualities that are required for field performance. The format of the assay involves the immobilization of an IFN-γ capture antibody onto plastic-backed nitrocellulose membrane using inkjet deposition. Then the sample is allowed to wick vertically up the membrane, which is facilitated by the attachment of a wicking pad to the nitrocellulose strip. Reagents to detect bound IFN-γ are then wicked up the membrane and the printed lines of the capture antibody binding IFN-γ antigen are developed. Thus far, this assay is sensitive to 1ng/ml IFN-γ. When used in combination with skin testing, this IFN-γ assay may prove useful in the diagnosis of early mycobacterium infection, and potentially other infections that induce a CMI response.

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INTRODUCTION

As part of the primary defense mechanism of the immune system, interferon gamma (IFN-γ) is a key protein in the immune response to infectious agents in vertebrates. Interferons are potent proteins produced by lymphocytes, macrophages, fibroblasts and epithelial cells[1]. We now know that interferon gamma is produced by T-lymphocytes (TH1 cells). T-lymphocytes are one type of lymphocyte produced by the immune system to help fight infection. Upon infection, the immune system's preliminary response is to recognize the antigen or immunogen and prevent it from spreading throughout cells of the body[2]. IFN-γ assists in doing this by either activating cytotoxic T-lymphocytes (CTL) to cause viral infected cells to undergo apoptosis or by causing macrophages containing bacteria to undergo a process inactivating the internalized bacterium[3].

Because of their function early in immune responses, detection of interferon levels has been explored as a way to diagnose diseases[4]. Diseases, such as tuberculosis (TB) among people and animals, can be diagnosed by testing for the production of interferon gamma by the immune system after stimulation with the antigen[5]. From a global health standpoint, the ability to detect disease incidence is critical in an effort to control and prevent a high prevalence. TB is one of the most commonly studied diseases in which infection leads to the production of high levels of interferon gamma[6]. It accounts for one of the leading causes of death in developing countries and is widespread throughout the world, especially in places where zoonotic TB persists[7].

Zoonotic infection is an ongoing challenge with *Mycobacterium bovis* (*M. bovis*) and will require a highly definitive prevention and eradication program. *M. bovis* is

carried by cattle, as well as other animal species, who often times are primary risk factors for the transmission of zoonotic TB and other zoonoses to people[7]. Other strains of TB, such as *Mycobacterium avium paratuberculosis* (*Map*), the cause of Johne's disease, are potential zoonotic factors, but also have a huge economic impact on the dairy industry[8] Johne's disease alone costs the U.S. over $220 million/year taking into account that there are no third party payers in veterinary diagnostics for food animals and profit margins in animal agriculture are small[8-10].

One of the biggest challenges in detecting infection in dairy cattle is that they do not often show signs or symptoms of the disease until months to years later and sometimes there are no symptoms whatsoever to be seen among people and animals[7]. This is the case for *Mycobacterium*, thus rendering it a primary target for detecting early infection. Knowing that IFN-γ is a key indicator of infection in the body, suggests that its detection may be used in efforts to control and eradicate disease. Current IFN-γ assays are effective in quantifying its levels in infected animals and people[6, 11, 12]. The genus of *Mycobacterium* is found in lymph nodes and can be detected by multiple serological and nucleic-acid based techniques[3, 13, 14]. In dealing with the recent issue of inadequate detection methods for Johne's disease in cattle, a low-cost, semi-quantitative and highly sensitive assay would be realistic for the detection of IFN-γ to achieve early diagnosis of the disease[15]. In combination with the IFN-γ assay, recent recommendations of performing a skin test followed by an IFN-γ detection assay would give optimal results for the diagnosis of early infection of *Map* and *M. bovis*[16, 17]. However, although some of the tests are simple enough to be able to be done in veterinary or medical clinics, in general, they require sophisticated laboratory equipment,

skilled laboratory technicians and furthermore they end up being quite costly[18]. The development of a low-cost, semi-quantitative, lateral flow, antigen-specific IFN-γ assay that is suitable for field use, may allow early detection of IFN-γ, thus contributing to the implementation of food-animal biosecurity for the protection of good public health. Maximizing test sensitivity is the biggest challenge for CMI responses to disease due to the biology of infections that cause this type of immune response, such as *M. bovis* and *Map*. The goal will be to improve the level of detection compared to the currently available assays, in order to detect IFN-γ found in the early stages of infection when disease symptoms are not apparent.

REVIEW OF THE LITERATURE

Discovery and function of interferons

During the 1950's, the field of virology became prominent because of efforts to control the poliomyelitis epidemic. The rapid breakthroughs in virological scientific knowledge led to the discovery of interferons in 1957[1], when Isaacs and Lindenmann observed that fluids from virus-infected cell cultures contained some factor that could react with cells and render them resistant to becoming infected[1]. This discovery was the first indication that interferons had significant antiviral properties and other vital cellular and bodily functions[1]

The immune system recognizes foreign pathogens and functions to target them for eradication from the body. Interferon gamma (IFN-γ) is a cytokine produced by specific blood leukocytes during a cell-mediated immune response that helps to trigger innate and then adaptive immune responses of vertebrates[3]. This antigen-induced protein is important in priming the body for further immune responses, such as cytotoxic T-cell proliferation and antibody production. Thus, it is a marker for the earliest response to infections in vertebrates. It is the host cell's first and foremost form of defense that the host has[1]

The three families of interferons have a wide array of functions including antiviral action, cell growth inhibition, and immunoregulatory and hormonal interactions [1]. Several interferons exert their biological effects by means of the same mechanism, implying that they are evolutionarily related and have diverged from a common ancestral gene; however, the mechanisms of IFN-γ activity are different from that of other

interferon families[1, 3]. Although they may differ in their mechanistic actions, all of the interferons play crucial roles as inducers and inhibitors of the immune system during a primary immune response as well as other anticellular and antitumor actions[1]. Interferon function is a highly studied field among researchers, doctors and the public health sector of food security and safety agencies[19].

Significance of Interferon gamma production in immune responses to pathogens

Recently, measurement of IFN-γ levels in conjunction with skin testing using specific antigen, has been suggested as a useful diagnostic tool for infection with *Mycobacterium bovis* (*M. bovis*)[7] and *Mycobacterium avium paratuberculosis* (*Map*) in livestock[5, 11]. A study done in 1999 showed that the production of CD4 lymphocytes, or T-helper cells, increases during the early phases of *Mycobacterium tuberculosis* infection. The cell-mediated immune (CMI) response to *M. bovis* or *Map* includes CD4 lymphocytes which produce IFN-γ, so that increased IFN-γ levels may be useful in detecting infection much earlier in the disease process than assays typically used in the past. These CD4+ Th1 cells (T helper cells) activate macrophages to phagocytize cells containing intracellular mycobacteria, thus killing the organism[20]. Yang and Mitsuyama showed that significant increases in IFN-γ-producing CD4 T cells occur immediately following *M. bovis* bacillus Calmette-Guerin (BCG) viable immunization[21]. In addition, they proposed that high levels of IFN-γ would also be produced by protective T cells when an individual was confronted with other intracellular microorganisms such as *Listeria monocytogenes*. Furthermore, the administration of the BCG vaccine also induced natural killer (NK) cell-dependent IFN-γ production *in vitro*,

which is important in the generation of protective T cells against *L. monocytogenes*. The critical importance of IFN-γ production by T cells in protection from infection was further supported in an experiment where mice were immunized with live/viable BCG vaccine and then given anti-IFN-γ; the ability of anti-IFN-γ-treated mice to generate protective T cells was significantly impaired. Furthermore, the production of antigen-specific, IFN-γ-producing T cells was terminated after the adoptive transfer of splenic T cells from mice immunized with live BCG[21]

Other groups have investigated how to enhance IFN-γ production and function. A study by Silva demonstrated protective T cell proliferation and increased IFN-γ mRNA in plasmid DNA-vaccinated versus BCG-immunized mice; furthermore, the duration of IFN-γ production by protective T cells was prolonged[22]. Lymph node T cells from DNA-vaccinated mice produced higher levels of IFN-γ when responding to either hsp65, whole BCG antigen or stimulation with PMA and anti-CD3, irrespective of whether the cells were CD8+CD4- or CD4+CD8- [22]. IFN-γ production *in vitro* peaked to the highest levels after cells from mice vaccinated with BCG were exposed to whole BCG antigen, and involved CD8+CD4- and CD4+CD8- T cells[22]. High levels of IFN-γ were detected as early as eight days in viable BCG-immunized mice[21] and these levels were maintained when cells were later stimulated with whole BCG antigen for up to 15 months after vaccination[22].

It has been shown in previous studies that systemic CMI responses to *Map* involve the increased production of IFN-γ by T helper cells (CD4+ cells) between weeks nine and eighteen after infection[20]. Early detection of IFN-γ has been achieved in calves experimentally infected with *Map*. Local CMI occurred in areas, such as the

mesenteric lymph node (MLN) and ileal and jejunal Payer's Patches, in response to stimulation with *Map* antigen. This was observed by an increase in lymphocyte production within the stimulated lymph nodes. Specific local CMI was evident as increased production of IFN-γ by stimulated caecal MLN cells, which could be detected six weeks post-infection[20]

On the other hand, in another study, neonatal lambs did not produce high levels of IFN-γ after prolonged infection with *Map*, for up to eight weeks[23]. RT-PCR of IFN-γ mRNA and detection of IFN-γ protein by ELISA showed that the production of IFN-γ by mycobacterium-stimulated lymphocytes was surprisingly low in the infected gut-associated lymphoid tissue (GALT) sites. This observation seems paradoxical given the essential role of IFN-γ production in protective cellular immunity to BCG. One mechanism that may explain this is that an important function of IFN-γ during a CMI response to mycobacterial infection is its role in impairing granulocyte-monocyte colony-stimulating-factor (GM-CSF) dependent macrophage activation. Macrophages readily support mycobacterial growth. In order to terminate intracellular growth, infected macrophages must be activated to stop any further mycobacterial replication. GM-CSF activates monocyte-derived macrophages to inhibit the intracellular growth of mycobacterium, thus killing the microorganism. In support, Burmudez and Youg, observed a significant decrease in intracellular inhibition of mycobacterial growth when GM-CSF-activated macrophages were treated *in vivo* with IFN-γ. In contrast, macrophage deactivation did not occur when mycobacterium-infected macrophages were treated with GM-CSF prior to the treatment of IFN-γ[23]. Thus, it appears that one of the more important mechanisms that IFN-γ may mediate during early paratuberculosis

infection is the termination of GM-CSF-dependent macrophage activation, prohibiting macrophage-dependent mycobacterial growth[23, 24].

Diagnostic approaches utilizing Interferon gamma detection

Increased production of IFN-γ may be useful in detection and eradication protocols aimed at reducing the prevalence of *Map* infection among livestock [11, 24]. Several detection methods for IFN-γ have been achieved over the years. The current IFN-γ enzyme immunoassay (EIA) was developed to detect early infection of *M. bovis* in cattle, and has been used in many countries since 1988 in protocols to eradicate TB and limit the spread of infection in infected herds[25].

Other approaches to detection of infection utilizing specific *M. bovis* proteins have been investigated, however their use in serological and/or cellular diagnostic assays is greatly restricted due to the genetic diversity of the bovine immune response to *M. bovis* infection[26]. Another issue is that an animal may be infected without clinical signs of disease. During very early stages of infection or in latently-infected subjects, *M. bovis* and *Map* occur at levels too low to be identified by culture techniques. On the other hand, cellular immune responses are detectable in infected subjects at an earlier stage than the pathological changes caused by the disease, or before the bacterial load exceeds the numbers necessary to be able to culture the organism from tissue samples (Figure 1)[17].

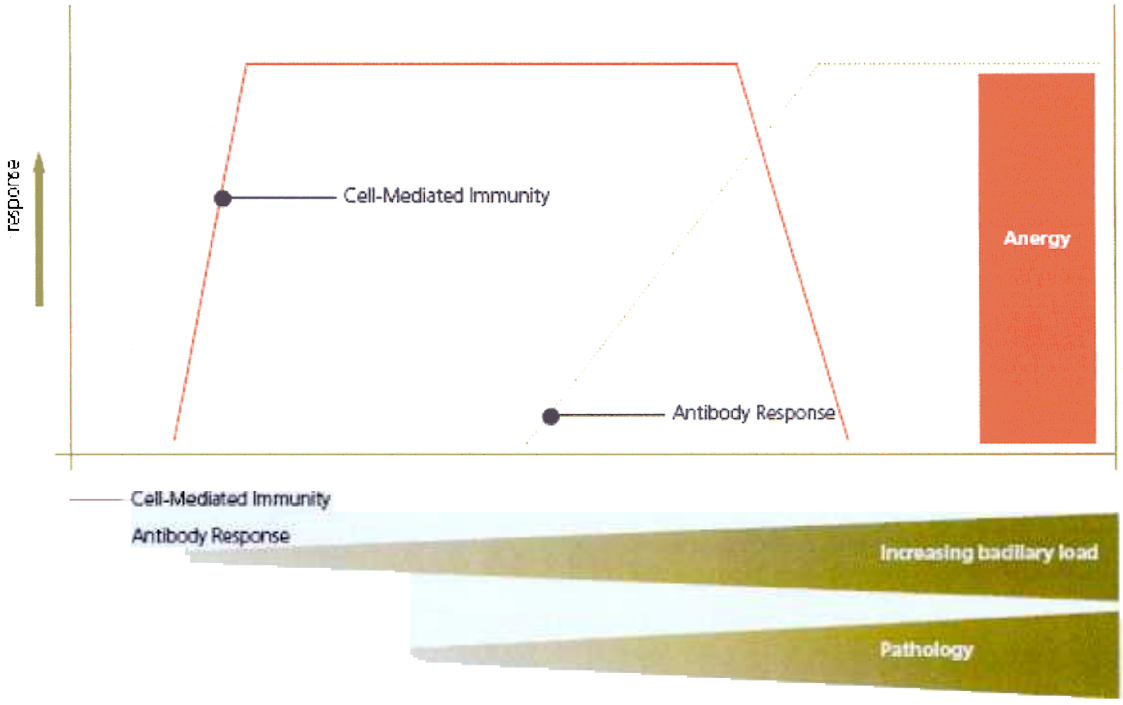

Figure 1. *Development of immune responses in cattle following M. bovis infection[17].*

Since antibody responses arise considerably later than CMI responses, it is not uncommon that early detection assays, like BOVIGAM™, are based on detecting IFN-γ[17]. Although the BOVIGAM™ assay has a relatively high sensitivity, ranging from 81.8% to 100%, and high specificity, between 94% and 100%, it is quite costly to perform[25]. Whole blood is required for this assay to be conducted and must be incubated with bovine purified protein derivative (PPD), avian PPD, or native control antigens in order to activate the cells to produce IFN-γ, which can then be quantified in separated plasma samples[26, 27]. Because it is an EIA, this assay also requires costly materials, like recombinant monoclonal antibodies, and a microplate reader. The time to result is 24 hours[26]. The BOVIGAM™ assay has also been reported to be effective in

detecting CMI responses to a wide array of other bacteria, parasites, viruses, various experimental antigens, T cell epitopes, vaccines and adjuvant formulations in cattle, sheep and goats[28]. While the most current IFN-γ assay is a blood test, and the testing procedures are more manageable for the farmer than skin testing, the test is certainly not suitable for point-of-care testing, where it would be most useful[26, 29].

The BOVIGAM™ assay detects mycobacterium infection more rapidly than another available test, the single intradermal tuberculin test (SIDT)[25]. The SIDT has been used to detect TB infection in many different species, including humans. It is very specific (96% to 99%) although it has significant cross-reactivity with other atypical mycobacteria. The SIDT has been used in conjunction with the BOVIGAM™ assay, and a study comparing the SIDT and the IFN-γ assay along with the standard ELISA to detect *M. bovis* infected goats concluded that the combination test may be more useful[16]

Detection of *Map* infection

Without a treatment or vaccination program in place in the United States that effectively prevents *Map* (Johne's disease), minimizing exposure to feces from infected animals in dairy herds by identifying and culling infected animals is the current method for controlling the spread of Johne's disease[9, 30, 31]. Serological methodologies involving the detection of *Map*-specific antibodies are available, yet they fail to detect the disease early on during infection[32, 33]. PCR testing methods for *Map* are also available, but they require a significant amount of time in order to isolate the organism through culturing techniques; they also demand specialized equipment and are

expensive[33]. Other attempts to develop assays for detection of *Map* infection have measured CMI responses using the tuberculin or Johnin antigen in conjunction with skin testing, or its *in vitro* equivalent, measurement of the IFN-γ levels with antigen stimulation. Both of these approaches have proven to be relatively non–specific.

However, as previously discussed, when IFN-γ detection is used in combination with skin testing, the specificity for detection of *Map* improves to 97.6% [15]. Thus, the development of an easy-to-use, low cost, semi-quantitative assay for IFN-γ would be highly beneficial to the animal production industry and public health. The use of this IFN-γ assay and skin testing in the field would greatly aid in efforts to control and eradicate mycobacterium, and potentially other infections that induce CMI.

Development of low-cost lateral flow-based immunoassays

Lateral flow point of care assays have become commonplace in drug testing, pregnancy testing, etc. and have been shown to be remarkably robust to the variation they are exposed to as home test solutions [34], and we chose this approach to develop a lateral flow assay for determining IFN-γ levels. In previous work, Lane and Gavalchin (unpublished) have used inkjet deposition to deposit antibody capture reagents on plastic-backed nitrocellulose because of its low cost and reproducibility, specifically, Millipore Hi-Flow Plus 180 membrane cards (60mm X 301mm), which have a ten mil plastic backing. This backing provides rigidity when the membrane is cut into strips. The test strip size is such that a total of 100 assays can be printed per 81/2 X 11 sheet of nitrocellulose.

An actual membrane strip, both before flow detection steps (left) and post detection steps (right-with the wicking pad removed), is shown in Figure 2. In the "lateral flow" approach, reagents were allowed to wick vertically up the membrane. Non-specific binding can be reduced by the addition of 0.5% casein in a rinse step prior to adding the detector conjugate, as shown in this trial. (Figure 2)

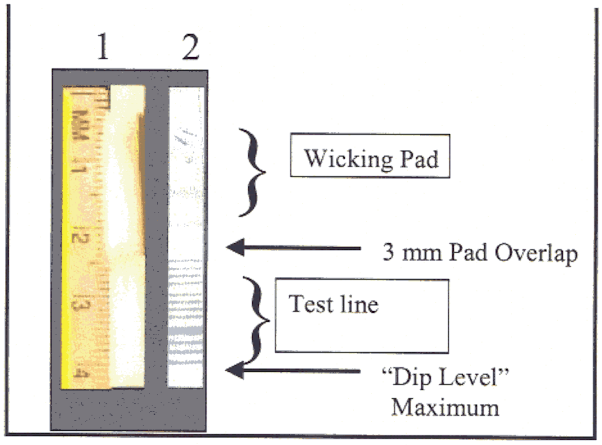

Figure 2. *Actual test strip before (1) and after (2) detection steps. On the left is an assembled strip with wicking pad (8 strips of Whatman 3MM paper, capacity ~700 µL) to facilitate flow of reagent vertically up the membrane. Strip was preprinted with biotinylated goat IgG at 3.2 µg/µL in TBS. The strip was first placed in a flat bottom vessel containing 200 µL TBS +0.5% casein. After that fluid was depleted, the strip was moved to a vessel containing 200 µL TBS + 0.05 µg/µL streptavidin:AP conjugate, followed by a 100 µL wash step in TBS. Total time for these steps is currently 45 minutes. The strip was allowed to dry and then immersed in BCIP/NBT for ten minutes. The reaction was stopped by immersing the strip in 1 mL dd H2O. The strip was then scanned on an HP flatbed scanner.*

The actual format of the field assay envisioned could look like that shown below in Figure 3.

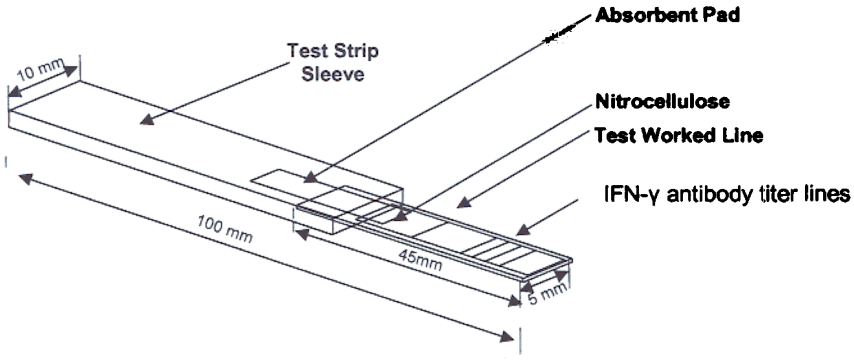

Figure 3. Lateral Flow IFN-γ Assay Design.

In essence, the test is a "dipstick" test that requires that the membrane "stick" be dipped into a diluted whole blood or serum sample. The sample will then flow up the nitrocellulose membrane printed with the IFN-γ antibody. The key features are a series of four identical anti-IFN-γ antibody capture lines, followed by a gap and a "test worked" line. By determining how many lines are positive, ie reacting with substrate, the titer of IFN-γ antibody in the sample can be determined. Based on other lateral flow assay results, the assay should be completed within 15-20 minutes [35].

The proposed IFN-γ assay has great potential for the detection of early infection of mycobacterium as well as other foreign antigens that induce a CMI response. Past evidence has shown that there is a real need for a sensitive, low-cost and quantitative IFN-γ assay, and we believe that the assay developed herein has these features. The versatility of this assay's design makes it adaptable to detect a wide variety of foreign pathogens causing CMI. This assay will be low-cost in performance and high in reproducibility. Additionally, it will be easy-to-use, quick to perform and suitable to use in the field. It should prove to be useful in TB and *Map* control and eradication programs worldwide.

MATERIALS AND METHODS

1. Immunization of rabbits and purification of rabbit anti-Bovine IFN-γ

The production of polyclonal anti-Bovine IFN-γ antibody was performed as previously described[36-38]. Briefly, one NZW rabbit was immunized subcutaneously with 75μg of bovine IFN-γ (R & D Systems, Minneapolis, MN) emulsified in Hunters Titermax Gold adjuvant (CytRx, Norcross, GA). Rabbit serum containing anti-IFN-γ IgG was obtained at 8 weeks post-immunization. The IgG fraction of the serum was purified by Protein G affinity chromatography (Pierce, Rockford, IL). Rabbit serum was adsorbed onto the Protein G column, and bound IgG was eluted using 3M urea. The IgG-containing fractions were dialyzed into PBS and then concentrated using a Centricon 30 filtration unit (Amicon, Billerica, MA).

2. ELISA for anti-Bovine-IFN-γ activity

ELISA was used to determine the specificity and titer of the purified anti-IFN-γ IgG. Briefly, recombinant bovine IFN-γ at a concentration of 23μg/ml in 0.05M carbonate buffer (Pierce, Rockford, IL) was coated onto the wells of a high-binding irradiated polystyrene microplate, Immulon 4 HBX (Dyanatec, El Paso, TX) in a volume of 50μl/well. The plate was covered with parafilm (VWR, Batavia, IL) and incubated overnight at 4C. The plate was washed twice with 200μl/well of PBS containing 0.1% PBS (PBS-Tw) (Sigma, St. Louis, MO) and then twice with PBS. A blocking buffer,

consisting of PBS containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO), was added to each well in a volume of 50µl/well. The plate was then covered with parafilm and incubated for one to two hours at room temperature, after which the plate was then washed as was previously described. Purified anti-Bovine-IFN-γ IgG was diluted at a 1:25 dilution in PBS-Tw, and then diluted serially 1:25 to the last dilution, :25600. The dilutions were added to the plate in quadruplicate at 50µl/well. The plate was covered with parafilm and incubated overnight at 4°C. The plate was then washed as was mentioned previously. Anti-rabbit IgG conjugated to HRP (Serotec, Raleigh, NC), diluted 1:500 in PBS-Tw, was added at 50µl/well. The plate was then covered with parafilm and incubated overnight at 4°C. After washing again, Tetramethylbenzidine substrate (TMB) (Sigma, St. Louis, MO) was added at 50µl/well. A dark blue color developed and the reaction was stopped by the addition of 50 µl/well of 1M H_3PO_4. Color development corresponding to bound IgG was then quantitated using an automated microplate reader at UV wavelength of 450nm, Genios Multi-Detection Microplate Reader with Magellan software (Tecan, Durham, NC).

3. Optimization of the IFN-γ dipstick assay

A. Printing of anti-IFN-γ capture antibody

The first step in the development of the IFN-γ assay was to optimize the amount of polyclonal anti-IFN-γ antibody bound to the membrane strip for assay. To do this, anti-IFN-γ was printed onto Millipore Hi-Flow Plus 180 membrane cards (60mm X 301mm) (Millipore, Billerica, MA) using an HP DeskJet printer (DeskJet Model 3945).

Serial dilutions of the antibody, 1:100, 1:1000 and 1:10000 in TBS (Sigma, St. Louis, MO), were printed in a block pattern so that each dilution was a different block in sequential order. All dilutions were printed, in order of concentration, as lines on three nitrocellulose strips. The efficiency of printing was monitored by the inclusion of trace quantities of yellow food dye (Butler' "Food Colors", Manchester, CT). The nitrocellulose membrane cards were then cut into 5mm X 45mm size strips to be used as individual assays

B. Detection of printed anti-IFN-γ antibody

The individual strips were placed in 200µl TBS containing 0.5% casein (TBS-casein) (Sigma, St. Louis, MO) for 15 minutes to block non-specific binding, and then removed and air-dried. A wicking pad made of Whatman 3MM paper (capacity ~700 µl) (Millipore, Billerica, MA) was attached to each of the nitrocellulose strips using tape, in order to facilitate the flow (wicking) of reagents up the membrane in subsequent steps The strips were then inserted vertically into wells containing 100µl of a 1:80 dilution in TBS of biotinylated rabbit IgG (Serotec, Piscataway, NJ,). After the solution was completely wicked onto the membrane, the wicking pads were removed and each strip was washed with 100µl of TBS for 15 minutes. A new wicking pad was placed onto each strip and Strepavidin-alkaline phosphatase (AP) (Pierce, Rockford, IL) was wicked, again in a volume of 100µl/well, at a dilution of 1:2000 in TBS. The wicking pad was removed and the strips washed as previously described. BCIP/NBT substrate for detecting AP (Trinity Biotech, Carisbad, CA) was added and bound AP visualized by the development

of a blue-black color. Proteinase-K (Sigma, St. Louis, MO), at a dilution of 1:10 in TBS, prepared from a stock with greater than 500 units/ml, was then added to stop the reaction.

C. Capture of IFN-γ by printed anti-IFN-γ antibody

The next experiment tested whether the printed anti-IFN-γ capture reagent bound to IFN-γ. Based on the results of the first printing experiment (above), a 1:1000 dilution of the anti-IFN-γ antibody in TBS was printed onto a Millipore Hi-Flow Plus 180 membrane card in a pattern of 12 lines, which was then cut into strips. The strips were then blocked in TBS-casein. Four dilutions of recombinant IFN-γ were made from a 25μg/100ul stock (in TBS) and allowed to wick up each of the four individual membrane strips at the following concentrations, 8ng/100μl, 4ng/100μl, 2ng/100μl and 1ng/100μl. Once the solution was completely wicked up each of the membrane strips, the wicking pads were removed and the strips were washed in TBS. A new wicking pad was placed on each strip and 100μl of mouse anti-bovine IFN-γ antibody (Serotec, Raleigh, NC), at a dilution of 1:80 in TBS, was wicked up the strips. The strips were then washed as described above and biotinylated anti-mouse IgG (Amersham Life Sciences, Piscataway, NJ), was wicked up each of the strips at a dilution of 1:80 in TBS. The strips were then washed again and Strepavidin-AP was wicked up the strips, at a concentration of 1:80 in TBS. Bound AP was detected with BCIP/NBT substrate. Color development was stopped after 15 minutes with Proteinase-K as above.

D. Optimization of anti-mouse IgG-biotin antibody

The anti-mouse IgG-biotin reagent was next optimized. Rabbit polyclonal anti-IFN-γ antibody was printed onto a Millipore Hi-Flow Plus 180 membrane card at a dilution of 1:1000 in a pattern of 12 lines. The strips were blocked with TBS-casein. The limit of detection for IFN-γ was established in the previous assay (C, above) to be between 1 and 2ng/100µl. Therefore, these concentrations of IFN-γ were chosen for this assay. Three membrane strips were each wicked with 1ng/100µl IFN-γ and two strips were each wicked with 2ng/100µl IFN-γ. One strip served as a negative control and was wicked with 100µl of TBS. After washing, mouse anti-bovine IFN-γ antibody at a dilution of 1:80 in TBS, was wicked up all six strips. The strips were then washed with 100ul of TBS and either 100µl of a 1:100 or a 1:250 dilution of biotinylated anti-mouse IgG in TBS was wicked up the appropriate strips. The strips were then washed with TBS and Strepavidin-AP at a 1:100 dilution in TBS was wicked up all six strips. The strips were washed with TBS and then BCIP/NBT substrate was wicked to detect AP. Color development was stopped by the addition of Proteinase-K.

E. Optimization of capture and detector reagents

This optimization step of the assay reversed the order of rabbit and mouse anti-IFN-γ capture antibodies. Monoclonal mouse anti-bovine IFN-γ antibody was printed at a concentration of 1:100 in a pattern of 12 lines onto a Millipore Hi-Flow Plus 180 membrane. After blocking with TBS-casein and washing with 100ul of TBS, recombinant IFN-γ was wicked up individual strips at concentrations of 250ng, 25ng, 2.5ng/100µl or 0, as a negative control. The strips were washed in TBS at 100µl/strip and the wicking pads changed. A 100 dilution of rabbit anti-IFN-γ antibody in TBS was

wicked up each of the strips. The strips were then washed, new wicking pads applied, and a 1:80 dilution of biotinylated anti-rabbit IgG (Amersham Life Sciences, Piscataway, NJ) in TBS was wicked up each membrane strip. The strips were again washed with TBS and Strepavidin-AP was wicked up each of the strips at a dilution of 1:2000. BCIP/NBT substrate was added and color development stopped by the addition of Proteinase-K.

A second assay was performed as described above, but with recombinant IFN-γ wicked at concentrations of 25ng, 2.5ng, 0.25ng, 0.025ng or 0 in 100μl TBS. The polyclonal rabbit anti-IFN-γ antibody and the biotinylated anti-rabbit IgG concentrations used were decreased two-fold to dilutions of 1:200 for the rabbit anti-IFN-γ IgG and 1600 for the biotinylated anti-rabbit IgG.

The concentration of the anti-rabbit IgG-biotin reagent to be used was also further optimized. Mouse anti-Bovine IFN-γ antibody was printed at a dilution of 1:100 in a pattern of 12 lines onto a Millipore Hi-Flow Plus 180 membrane. Recombinant IFN-γ at a concentration of 25ng in 100μl was wicked up each of 5 individual strips. The strips were blocked in TBS-casein, washed in 100μl/strip TBS, and the wicking pads changed. Rabbit anti-IFN-γ antibody was then wicked up the membrane at a dilution of 1:100, 100μl per strip. The membranes were washed in 100μl/strip TBS and wicking pads changed. Serial two-fold dilutions of biotinylated anti-rabbit IgG, beginning at 1:100 and ending at 1:1600, were wicked up each of the individual membrane strips. The strips were again washed with TBS as above and then Strepavidin-AP at a dilution of 1:2000 was wicked up each of the strips. BCIP/NBT substrate was added and color development stopped by the addition of Proteinase-K.

RESULTS

Titration of rabbit anti-Bovine-IFN-γ

The binding of the polyclonal rabbit anti-Bovine-IFN-γ antibody to IFN-γ was determined by ELISA (Figure 4). There was high binding up to dilutions of 1:800 (Dilution 6 in figure 4), after which the binding decreased with increasing dilution. Based on this result, it was decided that a dilution of 1:1000 of polyclonal rabbit anti-Bovine-IFN-γ antibody would be printed for optimal detection of IFN-γ run in the lateral flow assay format.

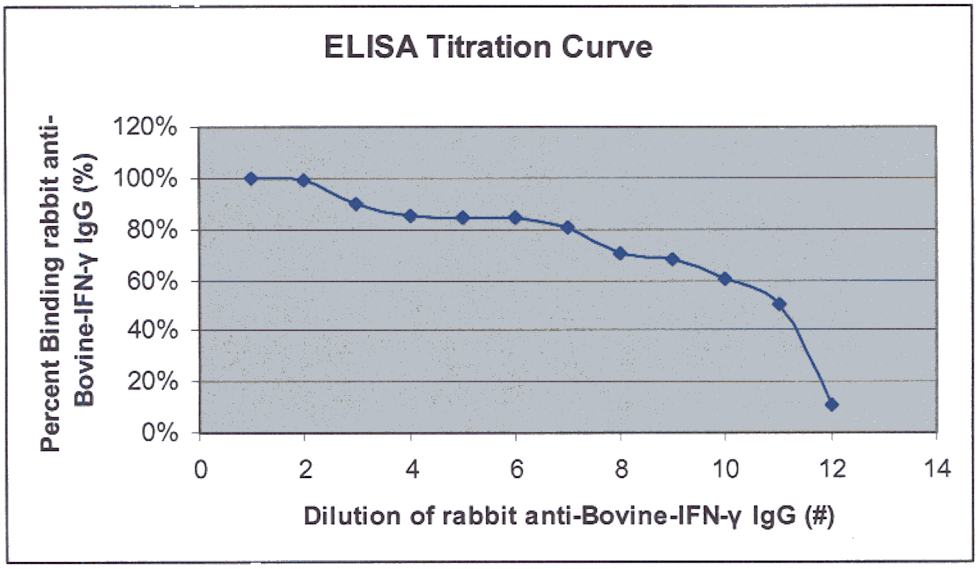

Figure 4. *ELISA titration curve. Polyclonal anti-Bovine-IFN-γ antibody was titrated in serial dilution of 1:25 to 1:25600 (dilutions represented as numbers 1 to 12, including the negative control) and plated in quadruplicate in wells coated wells with 50µl IFN-γ at a concentration of 23µg/ml. Detection was accomplished by the addition of anti-rabbit IgG conjugated to HRP at a dilution of 1:500. Binding of polyclonal anti-Bovine-IFN-γ antibody was reported as OD 450nm.*

Development and optimization of the lateral flow dipstick assay for IFN-γ

The IFN-γ assay was assembled by first immobilizing the capture antibody onto nitrocellulose membrane, using inkjet deposition and yellow food coloring as a flow marker, as seen in Step I of Figure 5. The membrane was then cut into 5mmX45mm assay strips (Step II) and blocked with casein containing TBS. After drying, all of the test trips were each placed vertically upright into a well with 100μl of IFN-γ and then detector antibodies to allow wicking of the reagents (Step IV). A top and side view of the test strip assembly with wicking pads is shown in Step III of Figure 5. Between each wicking step, the strips were washed and a new wicking pad applied. Bound IFN-γ was detected with the addition of BICP/NBT substrate (Figure 5).

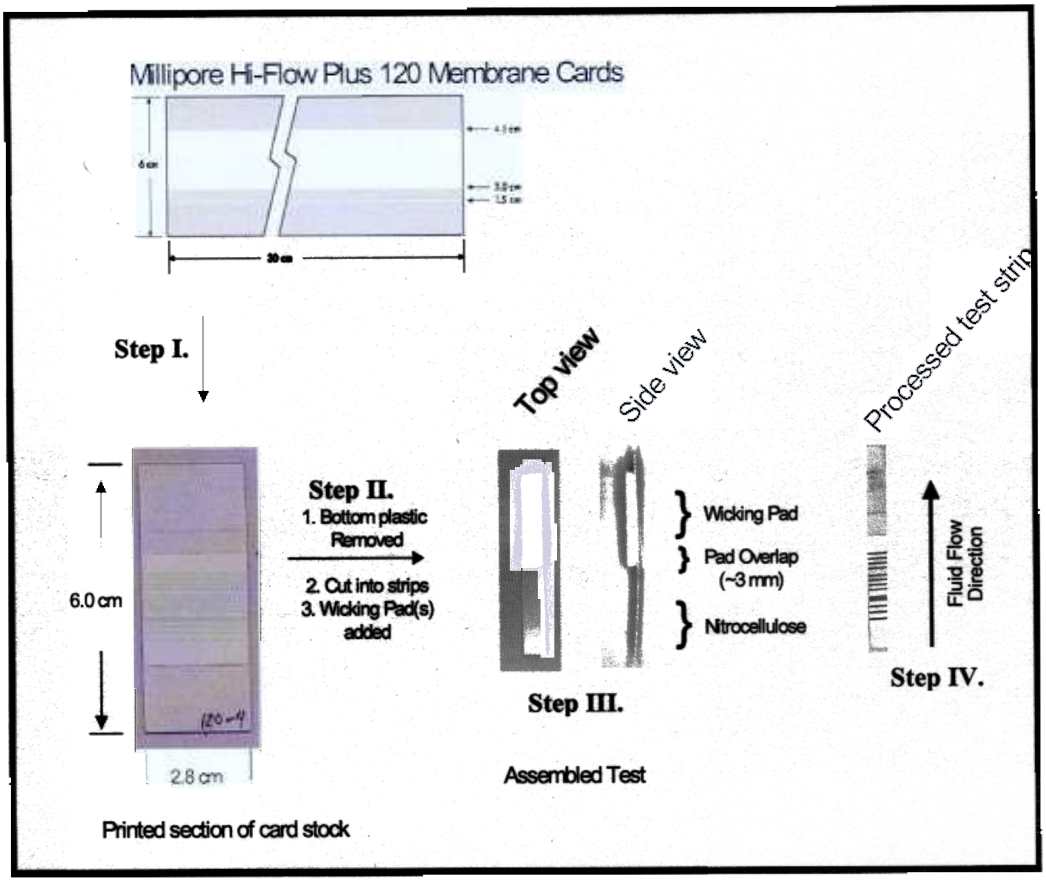

Figure 5. *Assembly of the IFN-γ assay. Step I. Nitrocellulose membrane card used to print capture antibody. Step II. (1)The printed section of card stock is then processed by removing the bottom plastic portion, (2) then the card stock is cut into strips 5mmX45mm in size, (3) and wicking pads were added to each strip. Step III. The final assembled test is seen in both a top and side view. The wicking pad overlaps the nitrocellulose membrane by ~3mm. Step IV. The processed test strip wicks reagent up the membrane vertically upwards.*

We first demonstrated that polyclonal rabbit anti-IFN-γ antibody could be printed and bound to membrane in the lateral flow assay format. Serial dilutions of 1:100, 1:1000 and 1:10,000 of anti-IFN-γ antibody were all detectable with a 1:80 dilution of

biotinylated anti-rabbit IgG, followed by a 1:2000 dilution of Strepavidin-AP. At the highest dilution of printed anti-IFN-γ antibody, 1:10,000 (C. in Figure 6), there was very little antibody detected. The 1:1000 titration (B. in Figure 6), was clearly detected by the anti-rabbit antibody. Binding at the 1:100 dilution was even stronger (A. in Figure 6).

Figure 6. *Detection of rabbit anti-IFN-γ antibody using lateral-flow, dipstick assay. Polyclonal anti-IFN-γ antibody was printed in serial dilutions of (A) 1:100, (B) 1:1000 and (C) 1:10000, in triplicate. Detection of the printed rabbit antibody was achieved using a 1:80 dilution of biotinylated anti-rabbit IgG, followed by a 1:2000 dilution of Strepavidin-AP. AP was developed with BCIP/NBT substrate and the reaction stopped with Proteinase-K.*

To determine whether the printed polyclonal rabbit anti-IFN-γ antibody bound to IFN-γ, it was printed onto four nitrocellulose membrane strips by inkjet deposition (Figure 7), at a concentration of 1:1000, based on the results of the previous experiment, above. Four concentrations of recombinant IFN-γ were prepared: 8ng/100µl, 4ng/100µl, 2ng/100µl and 1ng/100µl. The IFN-γ was wicked up the membrane, followed by mouse anti-Bovine IFN-γ antibody, then biotinylated anti-mouse IgG. Strepavidin-AP was next

wicked and the last step was the addition of substrate. IFN-γ observed as 12 lines, was detected under all conditions, with the limit of detection at 2ng/100ml IFN-γ (strip 3 in Figure 7). However, background of non-specific binding was relatively high on all strips, suggesting that the concentrations of detector antibody, mouse anti-bovine IFN-γ antibody or biotinylated anti-mouse IgG used in the assay required further optimization. (Figure 7)

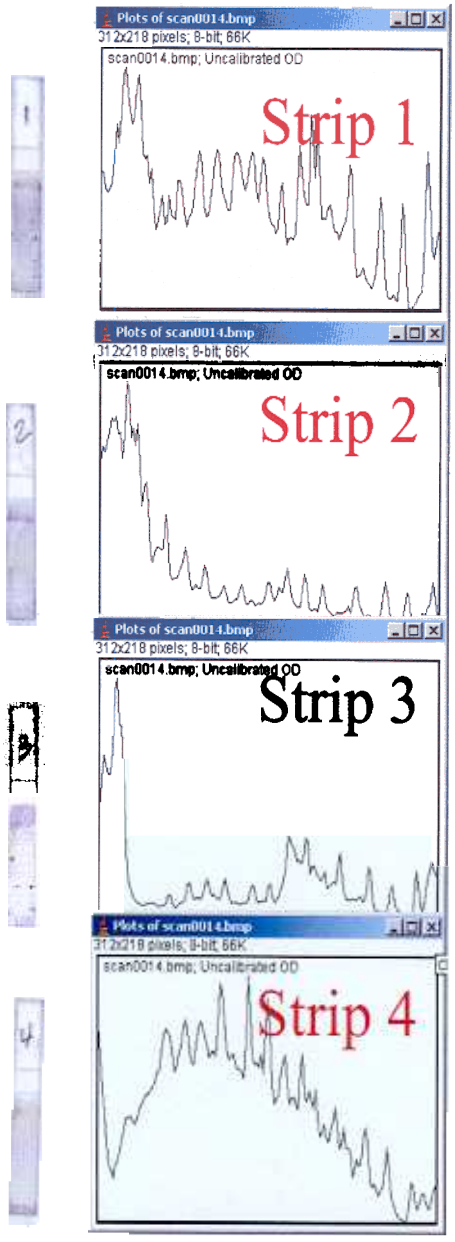

***Figure** 7.Detection of IFN-γ by rabbit anti-IFN-γ. Polyclonal rabbit anti-IFN-γ antibody diluted 1:1000 was printed onto the nitrocellulose membrane. Recombinant IFN-γ was added at 8ng/100µl, 4ng/100µl, 2ng/100µl and 1ng/100µl, with the limit of sensitivity of the assay at a concentration of 2ng/100µl.*

The following experiment was performed to optimize the anti-mouse IgG-biotin reagent. There was no detection of bound IFN-γ, after IFN-γ was wicked up the membranes at concentrations based on the previous experiment and varying concentration of detector antibodies used (results not shown). A negative control strip was included and did not show any signs of detection suggesting that there was a problem with the detector antibodies. Without any signs of developed AP seen on any of the strips, too much detector antibody could have caused non-specific binding to occur, which could have caused blocking of the epitope, preventing the binding of monoclonal antibodies to detect IFN-γ antigen. By reversing the order of printed capture antibody with the detector antibody, optimization of detecting antibodies could be done in order to reduce the incidence of non-specific binding.

In the next experiment, the order of the printing and detecting antibodies was reversed, so that the mouse anti-IFN-γ was printed and the polyclonal rabbit anti-IFN-γ was the detector. A control with no IFN-γ was also included. The level of background in this assay format was still relatively high. However, IFN-γ was detected, as shown by the dark grey lines present on all of the strips except for the negative control (Figure 8). It was apparent that the cause of the high background was the biotinylated anti-rabbit IgG. This reagent was titrated further in order to identify the optimal concentration of detection antibody that could still detect bound rabbit anti-IFN-γ, thus IFN-γ. (Figure 8)

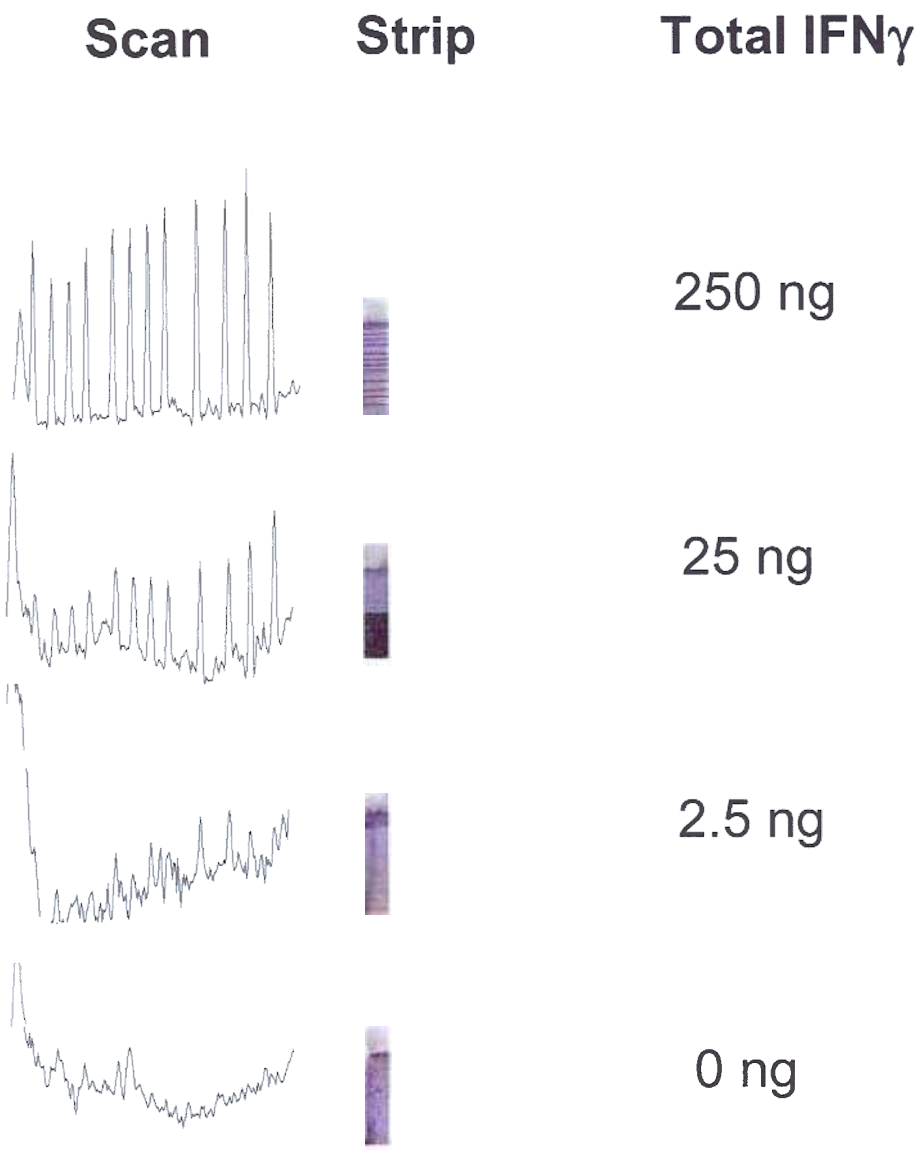

Figure 8. *Reverse format IFN-γ assay. Monoclonal mouse anti-Bovine IFN-γ antibody was printed at dilution 1:100. Recombinant IFN-γ of known concentrations: 250ng/100µl, 25ng/100µl, 2.5ng/100µl and 0ng, (Note: 2.5ng is 0.25ng here) could be detected in this assay format, with rabbit anti-Bovine IFN-γ antibody at a dilution of 1:100 and anti-rabbit-biotin at 1:80.*

The sensitivity of the assay was still maintained after the concentrations of the detector antibodies, namely rabbit anti-IFN-γ antibody and biotinylated anti-rabbit IgG were decreased, so that 1ng/100µl of IFN-γ could be detected. Still, background was high among all strips, including the control, shown in Figure 9.

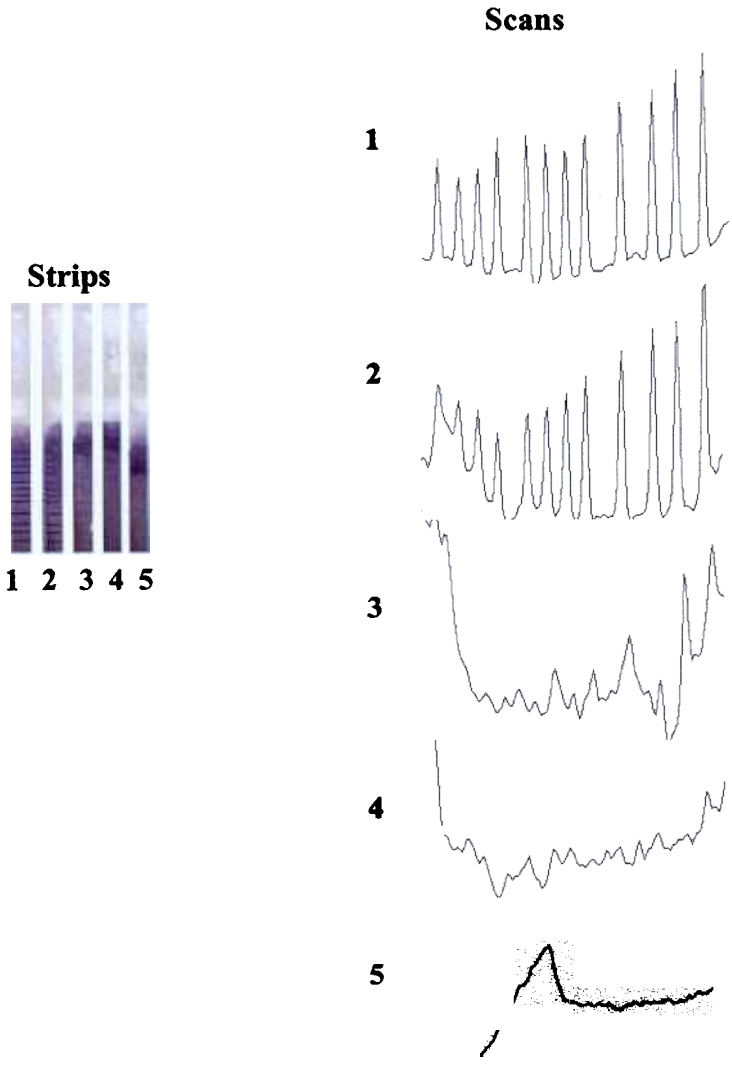

Figure 9.*Reverse format IFN-γ assay (trial 2). Monoclonal mouse anti-Bovine IFN-γ antibody was printed at dilution 1:100. Recombinant IFN-γ of known concentrations was detected: strip 1, 25ng/100µl, strip 2, 2.5ng/100µl, strip 3, 0.25ng/100µl, strip 4, 0.025ng/100µl and strip 5, 0ng. Rabbit anti-Bovine-IFN-γ antibody was used at a dilution of 1:200 and anti-rabbit-biotin at 1:1600. (Note: scans are reversed images of actual image)*

During the optimization of the anti-rabbit IgG-biotin reagent, all five strips assayed still showed high background, with the least amount at a dilution of 1:1600 (Figure 10).

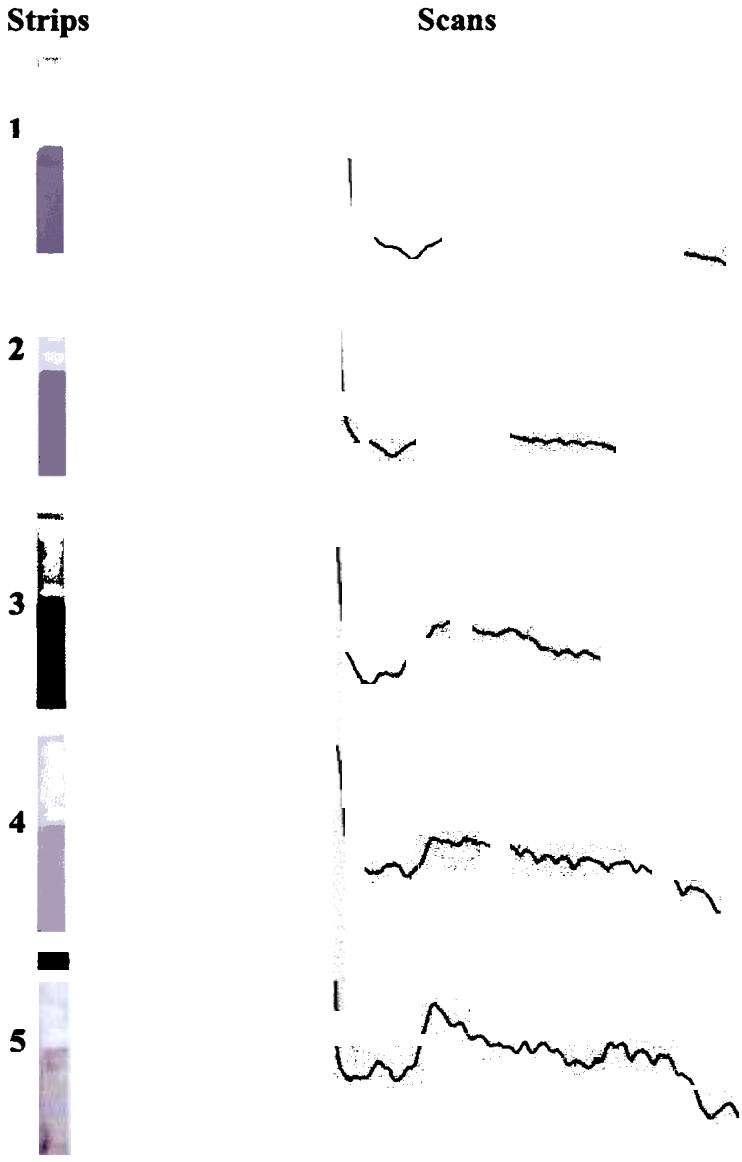

Figure 10. *Optimization of anti-rabbit IgG-biotin. Monoclonal mouse anti-Bovine-IFN-γ antibody was printed at dilution 1:100. Recombinant IFN-γ of known concentration was detected: 25ng/100µl. Rabbit anti-Bovine-IFN-γ antibody was used at a dilution of 1:200 and anti-rabbit-biotin at a dilution of 1:100 for strip 1; strip 2, 1:200; strip 3, 1:400; strip 4, 1:800; strip 5, 1:1600. (Note: scans are reversed images of actual image)*

DISCUSSION

In this study we present an assay format for the detection of IFN-γ that is low-cost, robust and sensitive, and potentially suitable for field use. Because there are no current assay methods available with these attributes, that are also both highly specific and sensitive, this assay could have a significant impact in the animal production industry, for example, in the diagnosis of *M. bovis* and *Map* infections. Current methods for detecting mycobacterium are very expensive and require trained individuals to run the costly and complex laboratory equipment required. While the assays are specific, they are not sensitive[17]. Previous studies have shown that skin testing for TB and Johne's in ruminants, in combination with IFN-γ detection, is both sensitive and specific[17], and thus may be a successful method to achieve early diagnosis of the disease. However, the current assay for detecting CMI-induced IFN-γ is species-specific and not quantitative; further it requires experienced personnel and specialized equipment. Cost and time-to-result are also prohibitive for its effective incorporation in herd management programs.

Over the past years, there have been many attempts to characterize the immune response to the *Map* antigen during early infection in cattle and prior to any obvious detectable symptoms, in order to develop a diagnostic assay. PCR testing methods for *Map* have been developed, but they require a significant amount of time in order to isolate the organism through culturing techniques; they also demand specialized equipment and are expensive[33]. The BOVIGAM™ assay for IFN-γ has been successful in identifying early infectious *Map* and *M. bovis* mycobacterium in cattle and other Bovidae; however, while it is sensitive, it is not specific[17]. On the other hand, detection of IFN-γ in conjunction with skin testing to specific antigens may prove useful in the

early identification of infected cattle. Early detection is critical since it would facilitate culling of infected animals before they exhibit clinical signs of disease, and thus protect the remainder of the herd from infection. The BOVIGAM™ IFN-γ assay when used to test for *M. bovis* or *Map* alone, has a sensitivity between 70% and 94%[17]. When the single intradermal comparative cervical tuberculin test (SICCT) is used solely, it has been shown to have a sensitivity of 65% to 80%[17]. However, sensitivity has been reported to be 88% to 93% when the tuberculin skin testing is used in conjunction with the BOVIGAM™ test to diagnose *M. bovis*[17, 27], and this is now the recommended method for diagnosing mycobacterium infection among Bovidae. Results are interpreted as positive when blood from infected cattle produce more IFN-γ after stimulation with bovine tuberculin or Johnin than after stimulation with avian tuberculin[15, 17, 27, 29, 39].

It would be very useful to be able to perform a skin test and detect IFN-γ in the field, and the assay developed here will be tested in this capacity in future work, using deliberately and naturally infected cattle, which are available from Yung Fu Chang at the Cornell College of Veterinary Medicine, Department of Population Medicine and Diagnostic Sciences. Blood sampling and preparation for optimal use in the assay will also be determined, as well as the reproducibility of results. Our proposed IFN-γ assay is sensitive enough to detect as little as 1ng/ml of IFN-γ. Whereas the current BOVIGAM™ IFN-γ assay is capable of detecting 80pg/ml[28]. We feel that the sensitivity of our assay can be significantly improved in future work using Epitype's proprietary technology for increasing binding, which relies on the construction of inexpensive "avidity" constructs previously employed by Dr. Michael Lane (SUNY Upstate Medical University) as "linear

amplification reagents" for both ELISA assays and to visualize DNA reactions on an ELISA plate without an amplification step (eg. PCR or enzyme)[40, 41] In brief, to effect this amplification the "detector antibody" is covalently linked to am oligonucleotide (oligo-dT_{35}) which is used to bind a long (several thousand bases) polydA molecule followed by the attachment of a signaling antibody:AP conjugate, which is attached to dT_{20} oligomers. The long polydA can accommodate hundreds of dT_{20} oligomers. Making the assumption that any IFN-γ antigen caught by the capture reagent can be detected, it is proposed that we will increase the apparent affinity of the anti-IFN-γ antibody by employing it as a polyvalent construction. As the number of these interactions increases[42, 43], the probability that all IFN-γ/anti-IFN-γ interactions will dissociate simultaneously becomes exceedingly small. We anticipate, based on previous work, that use of the amplification constructs could amplify the sensitivity of IFN-γ detection by anti-IFN-γ up to several thousand-fold.

We also must optimize the assay for serum samples, which is especially important since the market for this assay is the farmer in the field. The current methodology for this IFN-γ assay has only used TBS as a dilutent for all reagents. However, since we will be measuring IFN-γ levels in serum from whole blood samples, proteins in the serum may cause some difficulties with regard to the ability of the serum to flow vertically up the membrane.

We will also explore the use of this assay platform for the development of other diagnostic tests. The format of our proposed assay is flexible enough to allow for the detection of other infectious diseases. For example, this format would be useful in the detection of antigen-specific antibodies, for example, anti-Map, which is produced in

Johne's disease. Assays for this antibody currently exist (Parachek), however they are only sensitive enough to detect very high *anti-Map* levels which occur late in disease. Specific cell populations that are modulated in response to infectious agents would also be potential candidates for detection by our proposed assay platform. Future application of this assay format to aid in the monitoring of HIV patients by detecting CD4+ T-cell titers is currently underway. This project, funded by National Institute of Health, Small Business Technology Transfer Program (NIH STTR), aims to develop an alternative method to flow cytometry, which is not easily available in third world countries.

Diseases such as TB and *Map* are extremely costly to both the livestock industry and the nation as a whole. Johne's disease alone costs the U.S. over $220 million/year[8-10]. Current control and eradication programs call for expensive and lengthy procedures for diagnostic testing. For a dairy producer in the U.S. to have a herd tested for mycobacterial infection, it currently costs up to $35/test/cow[44]. We are aiming to create a robust diagnostic assay that can be produced and run, which includes necessary reagents and personnel, at a cost of less than $1/assay. Costs are kept low by the production of proprietary polyclonal antisera and printing polyclonal capture antibodies by inexpensive inkjet printer deposition. Because all of the methods that make up the construction and performance of our proposed assay would be in the public domain, expensive licensing of technology would not be required.

Another advantage of the IFN-γ assay that we have developed is the improved length of time-to-result that it provides. A study looking at the detection of *M. bovis* infected cattle and the time it took to have the blood samples tested in a laboratory, showed that a high incidence of false-negatives might occur in *M. bovis* positive cattle

due to the delay in time between sampling and assay[6, 27]. The assay that we propose would be done on-site and so should minimize the incidence of false negatives due to extensive handling time. Further, the assay that we propose should yield results more quickly than the BOVIGAM™ assay, which requires up to 24 hours to completion. In its current configuration, the IFN-γ assay that we have developed requires only 3 hours; with further optimization we hope that the assay may be completed in as little as 20 minutes.

Although our results were clearly positive for binding and detecting IFN-γ, the level of background was not acceptable. In future work, we will identify better blocking agents and will also determine whether the addition of protein or detergent to the TBS dilutent leads to reduced background. Other capture and detector antibodies will be developed and titers to be used optimized, as well. We will also compare this assay to assays that detect nitric oxide (NO) and tumor necrosis factor alpha (TNF-α), which have also been reported to prove useful as indices for the diagnosis of *M. bovis*[45]. NO is produced by mycobacterial-infected macrophages in a large enough quantity to be detectable and it has similar kinetics of production as IFN-γ[45]. However, we expect that IFN-γ detection will be the more optimal assay, since Waters, et al. found that the nonspecific production of nitrite, the measured oxidation product of NO that is produced by cultured cells from mycobacterial-infected cattle, is problematic to the development of an effective NO-based diagnostic assay[45].

Although in the preliminary stages of development, this lateral flow dipstick IFN-γ assay has shown promise that it could be useful for the detection of IFN-γ, particularly during the early onset of mycobacterial disease. This IFN-γ assay would also be easy-to-use, low-cost in performance, having a high reproducibility, and could easily be

performed in the field. Lastly, it is proposed to give highly sensitive and quantitative results. Its ability to be adapted for other diseases that induce CMI, in potentially any species, are features that would be highly beneficial to the livestock industry, by improving biosecurity measures and decreasing profit losses due to uncontrollable infectious disease.

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