Efficacy of Cytokine Flow Cytometric Assay to Differentiate Between Tuberculosis Infected and BCG Immunized Cattle.

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Key words: ABSTRACT:

Mycobacterium bovis is the causative agent of bovine tuberculosis, a disease that is increasing in developing countries. In addition to increasing economic losses, the rise in bovine tuberculosis poses a human health risk. There is an urgent requirement for effective strategies for disease eradication; this will likely involve vaccination in conjunction with current test and slaughter policies. A policy involving vaccination would require an accurate diagnosis of M. bovis-infected animals and the potential to distinguish these animals from vaccinates. Currently used diagnostic tests, the skin test and gamma interferon (IFN-γ) blood test, have a sensitivity of up to 95%. A further complication is that M. bovis BCG-vaccinated animals are also scored positive by these tests. We assessed the possibility of using the quantification of IFN-γ -producing CD4+T lymphocytes by Cytokine Flow Cytometric analysis of intracellular IFN-γ expression for discrimination of M. bovis-infected animals from BCG- immunized in Egypt. Heparinized blood was collected from 2 infected dairy cattle, 2 BCG-immunized cattle and 2 control cattle. Blood was stimulated in the presence of anti-CD49d, anti-CD28 (1 µg/ml) and activated with PPDb (20 ug/ml) and PPDa (20 ug/ml). PMA (50 ng/ml) and ionomycin (1μg/mL) were used as a positive control. Blood was incubated at 37°C in 5% CO2 for 6 hours. After 2 hours, Brefeldin A (10μg/mL) was added. Cells were stained with CD3, CD4, CD45R0, CD69 and IFN-γ antibodies. Two flow cytometer (FC) gating strategy were designed for data acquisition and data were analyzed using the De Novo Software. Significant numbers of IFN-γ expressing CD4+ T cells were detected following culture of heparinized blood from M. bovis-infected animals, but not from BCG –immunized with purified protein derived from M. bovis (PPDb). The finding suggested that this assay could allow the discrimination of BCG-immunized cattle from infected cattle. It is recommended to evaluate this discrimination technique on large number of animals to deduce dependable results.

1. INTRODUCTION

Bovine tuberculosis is a chronic infectious disease characterized by the formation of granulomatous lesions in different organs, mainly lungs and lymph nodes. Bovine tuberculosis causes significant economic hardship for livestock farmers with estimates of >50 million cattle infected worldwide, costing $3 billion annually (Roswurm and Ranney 1973.). In developing countries, Bovine Tuberculosis represent a major problem with a prevalence that could reach up to 10-15% of cattle herds in some parts of Africa (Daborn et al., 1998). Bovine tuberculosis is a threatening factor that restricts the growth in international trades of the dairy and meat industries wherefore its control and eradication is essential. Bovine tuberculosis produced by M. bovis is an important human zoonotic disease associated with consumption of dairy products contaminated with the bacilli, and to
labor risk (such as direct contact or droplet transmission) in farms or slaughterhouses (Grange et al., 2001). A conservative estimate would be that it causes 2% of the total pulmonary TB cases and 8% of extrapulmonary TB cases. Moreover, it is estimated that 60% of the human population live in countries where cattle undergo no control or only limited control for Bovine tuberculosis (Cosivi et al., 2007).

M. bovis is an intracellular pathogen of macrophages and other cells of the monocytic type. While the diagnosis of many other infectious diseases of animals relies on the detection of humeral (antibody) responses to the infectious agent, the predominant immunological response in M. bovis-infected cattle is affected by T-lymphocytes (Ritacco et al., 1991). This has pointed to the importance of cell-mediated immune responses (CMI) in bovine tuberculosis, and the importance of measuring CMI to allow early and accurate diagnosis of infection.

The only vaccine currently available for tuberculosis is M. bovis bacilli Calmette-Guérin (BCG). BCG remains the prototype vaccine against which to judge the efficacy of any novel vaccine strategies and is a useful model to aid the development of differential diagnostic strategies. The most promising vaccination strategies identified to date have mostly involved improving upon BCG vaccination rather than replacing it (Vordermeier et al., 2006). Although, it is not sure that in the future effective subunit vaccine strategies will be developed that could replace BCG vaccination (Skinner et al. 2011.). There has been a growing realization that ethical considerations make it imperative for novel vaccines to be given in addition to BCG rather than replacing BCG altogether, particularly when immunizing young ages. Thus, it is widely accepted that novel vaccine strategies should include BCG vaccination with a view to complementing and boosting BCG rather than displacing it (Vordermeier et al. 2006).

The two tests currently approved worldwide for diagnosis of Bovine Tuberculosis in cattle, namely the in vivo intradermal tuberculin test and the in vitro (blood-based) Gamma interferon (IFN-γ) assay. The former detects a delayed-type hypersensitivity reaction to the intradermal injection of tuberculin, whereas the latter detects the release of IFN-γ in whole blood cultures stimulated with tuberculin using a cytokine enzyme-linked immunosorbent assay (González Llamazares et al. 1999). It is well documented that both diagnostic techniques are only partially successful in distinguishing infected cattle from healthy, uninfected ones as BCG vaccination compromises Tuberculin-based diagnosis strategies and IFN-γ detection techniques (Hope et al., 2002). It’s important to say that the development of more specific diagnostic techniques capable of discriminating between infected and uninfected/vaccinated animals (differential diagnosis) is a prerequisite for the development of a vaccine against tuberculosis in cattle so that existing test and slaughter control strategies may still be carried out alongside vaccination without economical loss.

Although flow cytometry appears to be a mainstay of research in the many fields, it has not been used to its full potential in the Bovine Tuberculosis field. However, the number of studies using flow cytometry for exploring the immune response against Bovine Tuberculosis has been increasing in recent years. Recently, FC was used to monitor the changes in lymphocyte subset distribution in response to recombinant M. bovis-antigens. Antigen- stimulated T-cell and/ or B-cell subsets were examined by single-colour FC for differences in the percentages of CD4+, CD8+, CD3+, and CD25+ T lymphocyte subsets, as well as of γδ T cells in PBMC cultures from both shedder groups and healthy controls after simulation with each recombinant antigen (Shin et al., 2005, Whelan et al., 2011). These IFN-γ release assays (IGRAs) have shown limited use in high prevalent settings, as they are unable to differentiate between BCG
vaccinated and M. bovis-infected animals. In contrast to these commercial assays, IFN-γ detection by flow cytometry has an additional advantage in that it enables identifying the specific cell population responsible for the cytokine production (Won and Park, 2010). If coupled with additional cell markers, flow cytometry also enables the characterization of the cell population responsible for the IFN-γ secretion. Utilizing this strategy assessing the immune response difference between BCG vaccinated and M. bovis infected animals is possible. The aim of this study is the evaluation of the quantification of IFN-γ-producing CD4+ lymphocytes by Cytokine Flow Cytometric analysis of intracellular IFN-γ expression for discrimination of naturally infected cattle from BCG immunized in Egypt.

2. MATERIAL AND METHODS
2.1. Cattle used in this study
   a) Naturally Infected Cattle: A total of 1150 cross-breed dairy cattle from different private farms were tested by single intradermal comparative cervical tuberculin test (SICIT). 
   b) BCG Immunized Cattle: Two BCG immunized cattle were chosen from a previously immunized herd. BCG produced by the tuberculosis unit, department of Bacterial Diagnostic Research (Tuberculosis), Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. 
   c) Uninfected Control Cattle: Two non reactors were selected from a herd with no history for infection with Bovine Tuberculosis.
2.2. Antigens
Tuberculins (Bovine and Avian) used in the SCITT and purified protein derivatives (PPD-B and PPD-A) used in flowcytometric assay were supplied by the tuberculosis production unit, department of Bacterial Diagnostic Research (Tuberculosis), Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.
2.3. Samples
Heparinized blood samples were obtained from two naturally M. bovis-infected, two BCG-immunized and two Tuberculin test negative.
2.4. Postmortem Examination
Specific Postmortem examination was conducted on slaughtered tuberculin positive cattle (Palmer et al., 2007). Suspected tissue samples were collected for further bacteriological methods.

2.5. Bacteriological Isolation and Identification
Hexadecyl pyredenium chloride (HPC) was used for digestion and decontamination of the tissue samples. Isolation of M. tuberculosis complex was done on Lowenstein-Jensen medium (with and without pyruvate). The culture isolates were identified as M. tuberculosis and M. bovis on the basis of biochemical tests (Corner et al., 1988).

2.6. Whole blood culture
The CFC protocol employed was a modification of that described previously by Suni et al. 1998. Briefly, the whole blood was stimulated in the presence of anti-CD49d, anti-CD28 (1 µg/ml) and activated with PPDb (20 µg/ml) and PPDa (20 ug/ml). PMA (50 ng/ml) and ionomycin (1µg/mL) were used as a positive control. Blood was incubated at 37°C in 5% CO2 for 6 hours. After 2 hours, Brefeldin A (10µg/mL) was added.

2.7. Cell surface and intracellular cytokines staining
One hundred μl of leukocyte suspension containing one million viable cells were distributed in 96 well polystyrene V-shape bottom microplate containing 0.75 µg of each mAb mix (anti-bovine CD4, CD45RO and CD69 mAb) and incubated in the dark for 30 minutes on ice. Cells were subjected to 3 cycles of washing and resuspension in 200 μl of first wash buffer. The cell pellet was resuspended in Goat anti-mouse isotype specific antibodies and incubated in the dark for 30 minutes on ice. Cells were subjected to 2 cycles of washing and resuspension in 200 μl of second wash buffer. After the final wash, cell pellet was resuspended in 100 μl of Fixation/Permeabilization buffer for 20 minutes in the dark on ice. Cells were subjected to 2 cycles of washing and resuspension in 200 μl of Perm/Wash buffer. Cell pellet was resuspended in 100 μl of Perm/Wash buffer containing 0.1 μg of anti-
bovine IFN-γ FITC and incubated for 30 minutes in the dark on ice. Cells were subjected to 2 cycles of washing and resuspension in 200 μl of Perm/Wash buffer. After the final wash, cell pellet was resuspended in 100 μl of buffered formaldehyde 2% and stored at 4°C in the dark until acquisition of cells by flow cytometer. The Anti-bovine CD4, CD45RO, CD69, CD28 and CD49d mAbs used in this study were kindly gifted to us by Prof. Dr./ W. C. Davis, Department of Veterinary Microbiology and Pathology, Washington State University.

2.8. Flowcytometric Data acquisition

For flow cytometric acquisition of stained labeled leukocytes, a Becton Dickinson FACSCalibur flow cytometer equipped with argon and red lasers, a Macintosh 5 computer, equipped with Cell Quest software (Becton Dickinson Immunochemistry systems, San Jose CA) was used to collect data at the Flow Cytometry Unit, Department of Clinical Pathology, Faculty of Medicine, Tanta University. FCS express software (De Novo software, Thornton, Ontario) was used to analyze the data. At time of data acquisition, forward and side scatter parameters were used to distinguish granulocytes and mononuclear cells. Two gates were drawn gate 1 (R1) on small lymphocytes and gate 2 (R2) on large lymphocytes (R2) with exclusion of granulocytes and debris. Then the FC was instructed to collect 100.000 events within R1 or R2.

3. RESULTS

3.1. Results of Tuberculin Test

We tested the dairy farms with a history of Bovine Tuberculosis infection. The number of tested animals was 1150. The results of tuberculin skin test showed 15 (1.3 %) positive reactor animals.

3.2. Results of Macroscopic Examination of the Slaughtered Cattle

The 15 positive reactor animals were slaughtered and examined for the Postmortem lesions of tuberculosis. There was 11 (73.3 %) of visible lesions and 4 (26.6 %) of Non-visible lesions (Table 1). The lesions were classified as follows: 1 (6.7 %) generalized type. 5 (33.3 %) pulmonary type, 3 (20.0 %) mixed type (pulmonary and digestive) and 2 (13.3 %) digestive.

3.3. Results of Bacteriological Isolation

The results of the bacteriological isolation showed 10 (66.6 %) positive culture. Then the result of identification of the 10 positive cultured isolate by using different biochemical identification tests showed 9 (60%) M. bovis.

Table 1: Results of gross lesions:

<table>
<thead>
<tr>
<th>No. of slaughtered animals</th>
<th>Types of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pulmonary</td>
</tr>
<tr>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 2: Correlation between the isolated mycobacterium and the site of infection.

<table>
<thead>
<tr>
<th>Site of infection</th>
<th>Types of isolated mycobacterium (10)</th>
<th>negative culture (36)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.bovis (9)</td>
<td>Unidentified Slow Grower AFB” (1)</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Digestive</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mixed</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>General</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NVL.</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>9</td>
</tr>
</tbody>
</table>

*AFB: acid fast bacilli.

3.4. Results of flow cytometric analysis

Figure 1: Representative flow cytometric dot plots of intracellular IFN-γ in whole-blood cultures stimulated for 6 hs with PPD-b in control, infected and BCG immunized cattle. Expression of intracellular IFN-γ within CD4+ T cells was examined by flow cytometry. Percentages of cells in each quadrant are illustrated. (A): SSC Vs FSC, (B): Gating on CD4+ T cells

Figure 2: Chart shows the proportion means of IFN-γ-Producing CD4+T lymphocytes
4. DISCUSSION:

Although, most of national programs for the detection and eradication of Bovine Tuberculosis have relied on the detection of cellular immune response, they can't predict the disease status of the responding cattle. However, the ability of distinguishing M. bovis-infected cattle from immunized animals could be of a great value for the development of a vaccine against tuberculosis in cattle so that existing test and slaughter control strategies may still be carried out alongside vaccination without economical loss.

The result of the tuberculin test illustrates the prevalence of tuberculin reactors cattle in the tested cattle for this study. From a total of 1150 tuberculin tested cross-bred dairy cattle, 15 were found to be reactors with a prevalence rate of 1.3 %. The prevalence rate recorded in the present study is comparatively lower than that given by other investigators in Egypt as 6.9% (Lotfy et al., 1960), 26.5% (Guindi et al., 1965), 4.6% (El Battawy, 2008) and 2.2% (Nasr et al., 2008) and in the other countries of the world as 6.6% in Kenya (Waddington, 1965); 3.2% in Brazil (Oliveira et al., 1983), 7.4% in Zambia (Cook et al., 1996), 10.6 % in Spain (Gonzalez et al., 1999), 7.9%, 11.6% respectively in Ethiopia (Ameni et al., 2003), 2007, 8% in Chad (Borna et al., 2009) and 3% in Ethiopia (Tschopp et al., 2009). The achieved results, however, do not reflect the actual status of Bovine Tuberculosis in the tested cattle herd, due to the limited number of animals investigated in this study allotted in certain farms which does not allow firm conclusions to be made, in addition, there are many different factors which could cause false negative results and lowering the sensitivity of tuberculin test, for instance newly infected cattle may not react to the tuberculin test, as reactions have been reported to develop between 3 and 6 weeks post infection for most animals (Hope et al., 2005) this is called the pre-allergic phase. Also a state of anergy may develop in cattle with advanced or generalized TB and (temporarily) in animals subjected to stress (Pollock and Neill, 2002).

Regarding to the relationship between tuberculin reactor cattle and site of lesions as shown in (Table 1) the 15 slaughtered tuberculin reactor cattle showing visible lesions distributed as 1 (6.7 %) generalized type, 5 (33.3 %) pulmonary type, 3 (20.0 %) mixed type (pulmonary and digestive) and 2 (13.3 %) digestive. The frequency and severity of the lesions were higher in the thoracic lymph nodes than the mesenteric lymph nodes. This result agrees with results of previous studies, which reported that 90% of TB lesions occur in the respiratory system (Ngandolo et al., 2009). This is due to that the intensive, husbandry systems make the respiratory excretion and inhalation of M. bovis is considered the main route of animal-to-animal transmission (Smyth et al., 2001)

The total isolation rates of mycobacterium from carcasses of reactors with and without lesions were summed up in (Table 2). From a total of 15 carcasses, 10 were positive cultures with an isolation rate of 66.6%. Other investigators reported, however, lower rates as 29.1% (Gallo et al., 1983), 8.9% (Payeur and Marquardt, 1988). On the other hand, Chul Soon Choi, 1981 in Korea reported a much higher isolation rate amounting to 92.1%. These results depend mainly on the actual disease status present in the tested herd and to some extent on the technique used for decontamination of tissue specimens. Also, (Table 2) showed the isolation of 4 unidentified slow growers (6.4%) this revealed that the suspected postmortem lesions are not necessarily caused by M. bovis as reported by (Quinn et al., 1999) and others who mentioned that many of cattle could be infected with mycobacterium other than M. bovis that would not replicate in the M. bovis-selective culture medium (Milian et al., 2000).

The measurement of antigen-specific IFN-γ by CFC has been suggested as an extremely sensitive methodology for assaying antigen-specific cells in PBMC and whole blood (Hughes et al., 2005, Karlsson, et al., 2003, Maino and Picker, 1998, Suni et al., 1998). One major advantage of CFC is that it...
is not only allows the enumeration of responding cells but also provides information on the phenotypes of these cells. CFC also offers the possibility of measuring more than one cytokine simultaneously, which may increase the diagnostic potential. The majority of studies assessing the use of CFC as a diagnostic test utilized separated PBMC. However, Suni et al. (1998) described a modification using whole blood. The results obtained with whole blood were comparable to those observed using isolated PBMC preparations, and it was suggested that increased frequencies of responding T cells were observed in whole-blood cultures (Suni et al., 1998). The use of whole blood reduces costs and cell preparation time and eliminates the possibility of cell activation through separation techniques.

The failure to control the spread of Bovine Tuberculosis in cattle has many contributory factors including the limitations on the sensitivities of diagnostic tests. In addition, potential strategies for the use of vaccine in cattle, such as neonatal vaccination (Hope et al., 2005) and heterologous prime-boost vaccination (McShane and Hill, 2005), are limited by the fact that BCG-vaccinated animals test positive by both the skin test and the IFN-γ test which create an important requirement for the development of quick, sensitive diagnostic tests that can differentiate BCG-vaccinated cattle from M. bovis-infected cattle. Previously, it was shown that this differentiation may be achieved in assays using other antigens (van Pinxteren et al., 2000). However, reactivity to these antigens is not observed in all M. bovis-infected animals (Pollock and Andersen, 1997) and is significantly affected by prior sensitization of cattle to environmental mycobacterium (Hope et al., 2005). There is an important requirement for the development of new sensitive diagnostic tests that can accurately distinguish vaccinated subjects from those that are infected with virulent mycobacterium. In this study, we described the use of a developed CFC-based technique for the detection of mycobacterial antigen-specific IFN-γ-secreting CD4+ T-cells in whole blood.

We demonstrated that M. bovis-infected animals had significant high proportion of IFN-γ-secreting CD4+ T-cells of memory phenotype that stimulated with PPD-B, compared with that of BCG vaccinates or control as shown in (Figure 1 and 2). These results are in contrast to those assessing the potential of CFC for the diagnosis of M. tuberculosis infection in human. In a number of such studies where cells were stimulated with PPD, it was not possible to distinguish BCG-vaccinated patients from TB patients (Antas et al., 2004; Hughes et al., 2005; Tesfa et al., 2004; Tilley and Menon 2000.). For this reason, further study on large scale should be conducted to clarify this primary study results and whether this assay could allow an accurate discrimination of BCG-vaccinated cattle from infected one.

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