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Evaluation Rabbit Serum of the Immunodominant Proteins of Mycobacterium Avium Paratuberculosis Extracts

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Abstract*—M. paratuberculosis* is a slow growing mycobactin dependent mycobacterial species known to be the causative agent of Johne's disease in all species of domestic ruminants worldwide. JD is characterized by gradual weight loss; decreased milk production. Excretion of the organism may occur for prolonged periods (1 to 2.5 years) before the onset of clinical disease. In recent years researchers focus on identification a specific antigen of MAP to use in diagnosis test and preparation of effective vaccine. In this paper, for production of polyclonal antibody against proteins of *Mycobacterium avium paratuberculos*is cell well a rabbit immunization at a certain time period with antigen. After immunization of the animal, rabbit was bleeded for producing enriched serum. Antibodies were purification with ion exchange chromatography. For exact measurement of interaction, western blotting test was used that this study demonstrated sharp bands appears in nitrocellulose paper and specific bands were 50 and 150 KD molecular weight. These were indicating immunodominant proteins.

***Keywords*—**Paratuberculosis, Immunodominant, Western blotting, Ion exchange choromatography.

I. Introduction

MYCOBACTERIUM avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis in ruminants.

Paratuberculosis affects farmed ruminants such as cattle, sheep and goats. In the United States alone, Johne's disease accounts for US$ 220 million in annual loss to the agricultural economy [1]. However, because most *M. avium* subsp. *paratuberculosis* infections are pre-clinical and therefore may go undiagnosed for several years, the actual cost to producers is probably much higher. Despite its heavy economic burden on the agricultural industry, there are still no practical chemotherapeutic agents or efficacious vaccination programs against Johne's disease.

MAP is the part of large and diverse Mycobacterium family that is defined by their acid-fast properties, mycolic acid-containing cell walls, and high genomic C + G content (61–71%). The family currently counts more than 130 established and validated species and subspecies [2].

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MAP infections are usually acquired during calfhood via the ingestion of contaminated colostrum, milk, feed and water, and fecal shedding of organisms by infected cattle usually starts around 2 years of age [3].

JD is characterized by gradual weight loss, decreased milk production and diarrhea due to the chronic, progressive, granulomatous enteritis and lymphadenitis [4]. However, clinical signs of the disease do not typically develop for several years after initial infection because of the slow growing nature of the organism and the change to humoral immunity from cell-mediated immunization during the late stage of infection [5].

Although JD is a serious disease of cattle and other ruminants, there is no effective vaccine against it. The data available on the efficacy of current JD vaccines are variable [6], [7] and no vaccine is available that can completely eliminate viable MAP from the host [8]. Vaccines consisting of whole killed or attenuated MAP organisms reportedly provide partial protection by delaying fecal shedding and reducing the number of clinically affected animals [9]. Whole cell vaccines often cause local reactions at the injection site leading to granulomas and abscess formation and induce cross-reactions to intradermal tests for *Mycobacterium bovis* [10].

Additionally, the efficacy of these vaccines is unknown. Potential human exposure through inadvertent inoculation during use of modified live bacteria is also a considerable public health issue [11], [12].

Importantly, some studies have also linked M. a. paratuberculosis with Crohn's disease in humans [13], [14].

A possible association between MAP and Crohn's disease (CD) has been the focus of research for many years due to similarities in disease pathogenesis with JD in cattle [15]-[16]. Recent reports on the isolation of MAP from multiple ruminant and non-ruminant species, including humans, strongly suggests a possible association [17]. Also, advances in molecular and immunological diagnostic methods support an association between MAP and CD [14].

Thus, continuous control of the infection within cattle herds is necessary via changes in herd management [18].

In recent years, researchers focus on identification of a specific antigen of MAP to use in diagnosis test and preparation of effective vaccine. In this study we are looking for the immunodominant proteins to use as specific antigen.

II. Materials and Methods

A. Antibody Preparation

To prepare polyclonal antibody against MAP antigen, one New Zealand white female rabbit immunization at a certain time period with antigen and Freund's adjuvant.

Complete Freund's Adjuvant (CFA), a mineral oil containing a suspension of whole or pulverized heat-killed mycobacteria which is emulsified together with a solution of the antigen of interest to form a water-in-oil emulsion, is effective in potentiating cellular and humoral antibody responses to injected immunogens. Adjuvant activity is a result of sustained release of antigens from the oily deposit and stimulation of a local innate immune response, resulting in enhanced adaptive immunity. An essential component of this response is an intense inflammatory reaction at the site of antigen deposition, resulting from an influx of leukocytes and their interaction with the antigens. The use of CFA is an important biologic resource for investigators, which should be used responsibly and with care in order to avoid or minimize the adverse effects of excessive inflammation. CFA may result in local inflammation and granulomatous reactions at the site of injection, lymph node structural changes, chronic inflammation, skin ulceration, local abscess or tissue sloughing, diffuse systemic granulomas secondary to migration of the oil emulsion, adjuvant-related arthritis, and very rarely, chronic wasting disease.

For most applications, CFA is usually only necessary for the initial immunization, while Incomplete Freund's Adjuvant (IFA), which lacks mycobacteria, is the adjuvant of choice for subsequent immunizations.

After the final immunization, blood samples were taken from the rabbit and blood samples were collected and then were centrifuged in 4000 rpm. After immunization of the animal, rabbit was bleeded for producing enriched serum.

B. IgG Preparation

In this study for IgG preparation used ion exchange chromatography that relies on charge-charge interactions between the proteins in your sample and the charges immobilized on the resin of your choice. Ion exchange chromatography can be subdivided into cation exchange chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, in which the binding ions are negative, and the immobilized functional group is positive. Once the solutes are bound, the column is washed to equilibrate it in your starting buffer, which should be of low ionic strength, then the bound molecules are eluted off using a gradient of a second buffer which steadily increases the ionic strength of the eluent solution. Alternatively, the pH of the eluent buffer can be modified as to give your protein or the matrix a charge at which they will not interact and your molecule of interest elutes from the resin. If you know the pH you want to run at and need to decide what type of ion exchange to use paste your protein sequence into the titration curve generator. If it is negatively charged at the pH you wish, use an anion exchanger; if it is positive, use a cation exchanger. Of course this means that your protein will be binding under the conditions you choose. In many cases it may be more advantageous to actually select conditions at which your protein will flow through while the contaminants will bind. This mode of binding is often referred to as "flow through mode". This is a particularly good mode to use in the case of anion exchange. Here one could use this type of mode to bind up endotoxins or other highly negatively charged substances well at the same time relatively simply flowing your protein through the matrix. So antibodies were purification from serum with ion exchange chromatography.

C. Western Blotting Test

The western blot (sometimes called the protein immunoblot) is a widely accepted analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein. Gel electrophoresis step is included in Western blot analysis to resolve the issue of the cross-reactivity of antibodies.

The technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize.

For exact measurement of interaction, western blotting test was used that this study demonstrated sharp bands appears in nitrocellulose paper.

III. Result

A. IgG Preparation.

Preparation of IgG rich fraction from immunized rabbit sera by ammonium sulfate precipitation followed by DEAE ion-exchange chromatography and it resulted in a highly fraction (first peak). First peak was the main fraction and the second and third peaks were considered for further study (Fig. 1).

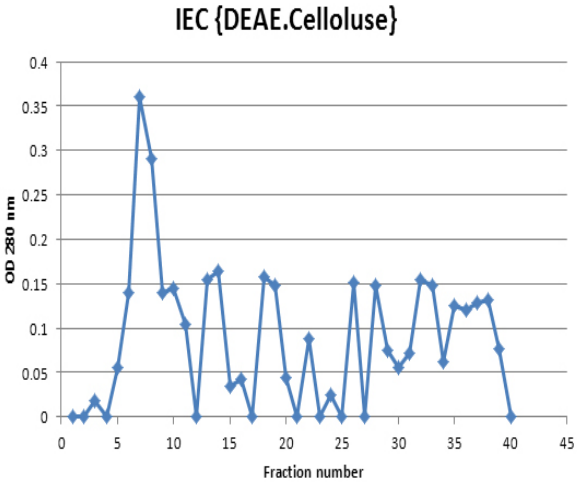

Fig. 1 IgG chromatography with DEAE – Cellulose column

B. Western Blot Analysis

Western blot on antigen at the dilution of 1: 10 with serum at 1: 15 dilution and immunoglobulin at 1: 10 dilution and IgG at 1: 2 dilutions of *M. avium* subsp were analyzed. Paratuberculosis which is a sharp bands appeared in nitrocellulose paper and these bands are of 50 and 150 KD molecular weight. These indicated immunodominant proteins (Fig. 2).

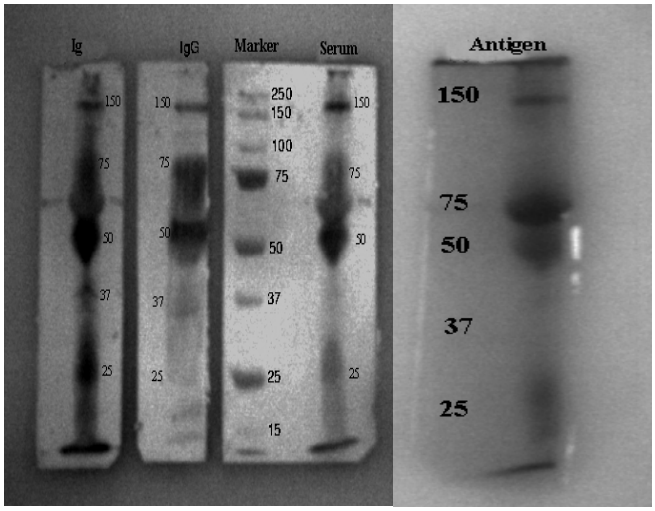

Fig. 2 Western blot analysis on antigen, serum, Ig, IgG

IV. Discussion

For the first time in 1985 the load of *M. paratuberculosis* antigen proteins were studied by Bech Nielsen et al. and proteins with molecular weight between the range of 42-28 KD were introduced as the region in the bacteria which has immunogenicity activity. But these scientists were not successful in observing the singular bands of the mentioned proteins in SDS-PAGE analyses [19].

A recent vaccination-challenge study identified the recombinant 70 kD heat shock protein (Hsp70) of MAP as a promising subunit vaccine candidate [20].

In 2010 a proteomic analysis approach, based on one dimensional polyacrylamide gel electrophoresis (SDS-PAGE) followed by LC-MS/MS, was used to identify and characterize the cell wall associated proteins of *M. avium* subsp. *paratuberculosis* K10 and an cell surface enzymatic shaving method was used to determine the surface-exposed proteins by Zhiguo He and Jeroen De Buck. 309 different proteins were identified, which included 101 proteins previously annotated as hypothetical or conserved hypothetical. 38 proteins were identified as surface-exposed by trypsin treatment. To categorize and analyze these proteomic data on the proteins identified within cell wall of *M. avium* subsp. *paratuberculosis* K10, a rational bioinformatic approach was followed. The analyses of the 309 cell wall proteins provided theoretical molecular mass and pI distributions and determined that 18 proteins are shared with the cell surface-exposed proteome. In short, a comprehensive profile of the *M. avium* subsp. *paratuberculosis* K10 cell wall subproteome was created. The resulting proteomic profile might become the foundation for the design of new preventive, diagnostic and therapeutic strategies against mycobacterial diseases in general and *M. avium* subsp. *paratuberculosis* in particular [21].

In this study in order to increase the stability and to prolong the process of antigens getting free the antigen was injected along with Freund's adjuvants. And finally in order to identify the dominant protein of antigens of the bacteria's body, Western Blot was carried out on the body of the bacteria in this study in which the related bands to immunodominant antigens were observed on the nitro cellulose paper as a result. Therefore by exploiting dominant antigens and better understanding these proteins from protein structure standpoint, more sensitive diagnostic test or even in preparing subunit vaccines to some extent could be used.

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