EVALUATION OF USING "LYSOZYME EXTRACT" ANTIGEN OF VIRULENT *MYCOBACTERIUM BOVIS* FOR DETECTION OF BOVINE TUBERCULOSIS

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SUMMARY

Lysozyme extract of Mycobacterium bovis was prepared from cells, which were previously treated with acetone, ethyl alcohol-ether, and chloroform. Cells were dried and suspended in Tris HCl, (pH 7.5) containing lysozyme and filtered sterilized.

It was found that lysozyme extract and mammalian PPD tuberculin, at equal protein concentrations, elicited comparable delayed- type hypersensitivity responses in cattle naturally exposed to M. bovis. ELISA results indicated that the values of sensitivity in mammalian, bovine PPD and lysozyme extract antigen were (84%, 88% and 92%), while the specificity was (71.4%, 85.7% and 85.8%), respectively. Accuracy of positive predication for each of the three antigens was (91.3%, 95.6% and 95.8%) and accuracy of negative predication (55.6%, 66.7% and 75%). Efficiency of predication was (81.3%, 87.5% and 90.6%) and finally, Error of predication was (18.7%, 12.5% and 9.4%) respectively.

These obtained results indicated that lysozyme extract antigen is better than PPD (mammalian and bovine) for minimizing non-specific reactions in diagnosis of bovine tuberculosis and could be used in diagnosis of bovine tuberculosis by means of skin test or measuring humoral immune response using ELISA.

INTRODUCTION

Bovine tuberculosis is a major worldwide disease with significant consequences for farming economy. The causative agent, is *M. bovis* one of the *M. tuberculosis* complex.

Many physiochemical approaches have been used to produce and purify mycobacterial antigens; however, definitive characterization of the components present in these antigen preparations is limited. Cultures filtrates and sonic or pressure cell-disrupted whole-cell preparations have been used to produce soluble mycobacterial extract (Daniel and Janicki, 1978). Two problems are not addressed in the preparation of biologically active mycobacterial extracts, the effects of endogenous proteases on mycobacterial components during growth and subsequent isolation and the effect of autoclaving on the component solubility and antigenicity in mycobacterial preparations.

Peptidoglycans are the basic cell wall constituents in all bacteria (Heymer and Rietschel, 1977; Schleifer and Seidl, 1977). Mycobacterial are different in the make up and composition of lipid, polysaccharide, and polypeptide components of their cell walls (Adam et al., 1973 and Lederer et al., 1975). Cellular and humoral immune responses to various lipid, glycolipid, polysaccharide and protein components of mycobacterial cell walls have been reported (Goren, 1982; Daniel and Janicki, 1978). Various species of mycobacteria differ in the composition and number of polypeptides in the cell wall (Wietzerbin et al., 1973).

Treatment of delipidated cell wall preparations of mycobacteria with lysozyme resulted in the release of water-soluble cell wall arabinogalactan peptidoglycan complexes, polysaccharides and low molecular weight complex of peptides (Adam et al., 1973 and Goren 1982). Peptidoglycan has adjuvanticity stimulates macrophages is mitogenic, and has antitumor and immunosuppressive activities (Lederer et al., 1975 and Goren, 1982), some polysaccharides and low molecular weight complexes of peptides have been used to inoculate guinea pigs and mice (Youmans, 1979).

So, the present work was designed to prepare a soluble filter sterilized lysozyme extract of *M. bovis* and use it as skin test antigen in natural infected cattle in comparison with the routinely used mammalian PPD tuberculin. Also using of this extract as an antigen for detection of mycobacteria antibodies in sera of cattle naturally exposed to *M. bovis* by using indirect ELISA technique.

MATERIAL AND METHODS

1) Antigens:

A. Lysozyme extract antigen:

It was locally prepared following the method of Hall and Thoen (1985) by growing *M. bovis* on Lowenstein-Jensen medium with sodium pyruvate. Cultures were incubated at 37°C for 12weeks. Colonies were scraped from the slants and suspended in sterile distilled water, then transferred to 250 ml centrifuge bottles and centrifuged at 10.000 xg for 30 minutes at 4°C. The supernatant was discarded and the cells were extracted three times with thirty volumes each of acetone, ethyl alcohol ether and chloroform (Lederer et al., 1975). The cells were air-dried and egg white lysozyme (Sigma Chemical Co.) was added to the dried cells at a ratio of 1 mg of lysozyme /50 mg of cells (Hall and Thoen, 1983). One gram of this mixture was suspended in 10ml of 0.05M Tris-HCl (pH 7.5) containing 0.003M EDTA and 10mM phenyl methyl sulfonyl fluoride, and then incubated by stirring for 16 hours at 37°C. Cells were removed by centrifugation, and the lysozyme extract was filter sterilized, using a 22-mm disposable filter unit (Hall and Thoen, 1983). Protein concentration of filter-sterilized lysozyme extract was determined according to Lowry et al. (1951). Extract was tested to be free from any bacterial or fungal contamination and dispensed in 2 ml aliquots and were stored at 4°C till used.

B. Mammalian and bovine PPD:

They were prepared by Bacterial Diagnostic Products Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

C. Mammalian tuberculin:

It was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. (2 mg / ml .)

2) Skin tests in cattle:

Comparisons of the prepared filter sterilized lysozyme extract and standard mammalian PPD tuberculin were applied in skin tests in naturally exposed cattle. The lysozyme extract was diluted in sterile phosphate-buffered saline solution (PBSS) 2 mg of protein /ml immediately before use. Intra-dermal test was conducted at separate sites (0.1 ml/site) on the shaved neck area of both sides. The injection sites were observed 72 hours after injection, and the skin thickness was measured by calipers. All the tuberculin reactor

cattle were slaughtered and examined carefully to detect any pathological changes including serial incision of all lymph nodes and internal organs. All samples were subjected to sample processing according to Marks (1972). Bacteriological isolation and identification were done according to Chadwick (1981).

3) Sera for ELISA:

Blood samples were taken from 32 skin reactor cows. Out of them, 25 showed tuberculous like lesions and the remaining showed no visible lesions by proper inspection of their carcasses at the abattoir and confirmed bacteriologically. Sera were taken, centrifuged and store at 20°C.

4) ELISA test procedure:

An indirect ELISA was conducted by modification of procedures described previously by Thoen et al. (1983) of which bovine and mammalian PPD and a filter-sterilized lysozyme extract of *M. bovis* were diluted to 10mg/ml in 0.1 M Na_2Co_3 (pH 9.6). An aliquot of antigen (0.05ml, 500 ng of protein) was added to each well of ELISA plate. In addition, 0.05 ml (1mg/ml) of carbodiimide, diluted in 0.1 M Na_2Co_3, was added to each well. The plates were incubated for 16 hours at 4°C, and then washed 3 times with PBSS. The plates were then incubated for 30 minutes at 22°C with 0.01 M NH_4Cl. (0.1 ml /well) and washed 3 times with ELISA washing solution (0.5 M NaCl containing 0.5% Tween 80, adjusted to pH (7.5).

Sera obtained from cattle were diluted 1:40 in ELISA diluent (0.5 M NaCl containing 1% bovine serum albumin and 1% tween 80, pH 7.5); 0.1 ml was added to the first wells of the plates. Double fold dilutions of serum were done by transferring 0.05 ml of serum to separate wells that contained 0.05 ml of diluent. Sera were incubated for 30 minutes at 22°C on a horizontal shaker. The plate was washed 8 times with ELISA washing solution and allowed to stand inverted for at least 30 minutes. Then 0.05 ml of an anti-Bovine Immunoglobulin G horseradish peroxidase, was added to each well and the plates were incubated for 30 minutes at 22°C on a horizontal shaker. The plates were again washed 8 times and allowed to stand inverted for 30 minutes. A working solution of hydrogen peroxide and 2,2`-azino- di (3 ethyl benzthiazoline-6-sulfate; ABTS) in 0.05 M citric acid (pH 4) was added to each well (0.15ml) and incubated for 60 minutes. The color intensity of the reactions was determined at a wave length of

405nm. A reaction was considered positive if it gave an OD value equal to or greater than the mean OD of the negative control samples plus two standard deviations (Nassau et al., 1976). A titre of 1:80 serum dilution or higher was considered as positive test for cattle serum (Morris et al. 1979).

5. Statistical analysis:

The evaluation of ELISA values was detected by accuracy index, sensitivity, specificity, positive and negative predictive values efficiency of predication and error of predication according to Daniel and Debanne (1987).

RESULTS

The soluble protein concentration of the prepared filter sterilized lysozyme extract antigen of *M. bovis* was found to be 2.2 mg/ml. Tables (1, 2 and 3) illustrated the results of skin test, ELISA and statistical analysis of ELISA reading.

With regarding to ELISA test reactions, the samples could be divided into; True positive that showing gross lesions, positive culture of *M. bovis* and positive ELISA. False negative which showing gross lesions, positive culture of *M. bovis* and negative ELISA. False positive that showing no gross lesions, negative culture of *M. bovis* and positive ELISA. True negative which showing no gross lesions, negative culture of *M. bovis* and negative ELISA.

DISCUSSION

Bovine tuberculosis is a disease of worldwide occurrence that causes great harm to dairy farms and health risks to the population that consumes products of animal origin. It is still a problem of both public health and economic importance in large areas of the world (Ritacco et al., 1987).

The standard diagnostic test for this disease is the intradermal tuberculin test. One of the oldest immunological tests still in widespread use (Wood and Rothel, 1994). It has a number of documented problems (Pritchard, 1988). The non-specific reaction is the most important problem when using PPD tuberculin. So, great efforts were performed to produce and evaluate new

reagents for recent diagnosis of bovine tuberculosis to overcome these difficulties (Daniel and Janicki, 1978).

In this study, it was attempted to produce an innovative antigenic extract, which is a lysozyme extract of *M. bovis* for diagnosis of bovine tuberculosis in naturally exposed cattle under field condition.

The protein concentration of the prepared antigen revealed that extraction of *M. bovis* with lysozyme resulted in the recovery of 2.2mg/ml of soluble protein in the filter-sterilized lysozyme extract. This result nearly similar to Hall and Thoen (1985) who recovered 2.7mg/ml of soluble protein in the filter sterilized extract.

The results depicted in table (1) showed that the two antigens could be able to differentiate between the infected and non-infected groups. These results proved that lysozyme extract of *M. bovis* could stimulate delayed type hypersensitivity (DTH) reaction similar to the mammalian PPD tuberculin and more sensitive than PPD tuberculin and could minimize the false negative cases as out of 650 examined animals, 32 cases gave positive reaction with lysozyme extract antigen while 31 animals gave positive reaction with mammalian PPD tuberculin. These animals gave visible lesions and positive cultures to *M. bovis* in bacteriological examination. These findings agree with results given by Hall and Thoen (1983), Gad El-Said et al., (2001) who found that a filter sterilized lysozyme extract of *M. bovis* was a potent skin test antigen in sensitized guinea pigs. This may be due to the component mediated the delayed type hypersensitivity (DTH). Responses in PPD may be different from these in a filter sterilized lysozyme extract (Hall and Thoen, 1985). Moreover, Daniel and Janiki (1978) found that the lack of sensitivity of PPD might be due to that it was a crude mixture of a highly variable composition. Daniel (1980) found also that PPD is a non-uniform product, which varies considerably in composition and activity.

ELISA technique applied as a sensitive method for measurement of antibody titre in sera of skin reactor animals (Engvall and Perlmann, 1972) for serodiagnosis of tuberculosis. Lysozyme extract antigen, bovine PPD and mammalian PPD were used in this work as coating antigens, the obtained results are displayed in table (2), which revealed that the lysozyme extract antigen is more sensitive and specific than the mammalian PPD

tuberculin. Hence, its ability to detect the cattle, which are infected with *M. bovis* and showing visible tuberculous lesions. These findings are supported by the results obtained by Hall and Thoen (1985) who proved potency of a filter sterilized lysozyme extract of *M. bovis* after exposure to *M. bovis* in experimentally exposed calves and that of Gad El-Said et al., (2001) who mentioned the superiority of lysozyme extract in distinguishing between guinea pigs infected with typical and atypical mycobacteria by using ELISA technique.

On the other hand, the true positive cases might have *M. bovis* antibody levels while the false negative groups (as shown in table 2) showed low ELISA values referred to insufficient antibodies present (Auer, 1987). The false positive ELISA reactions may be due to the effect of repeated tuberculin test as observed early 3 or more tuberculin test (Thoen et al., 1983) or due to non-specific reactions caused by IgM or IgA antibodies (Radin et al., 1983 and Yokomizo et al., 1983). While the negative ELISA test and absence of macroscopic findings may be due to low coincidence between tuberculin and ELISA tests (Ritacco et al., 1990).

Table (3) shows statistical evaluation of ELISA reading on sera using 3 different antigens. The higher values obtained by lysozyme extract antigen than with mammalian and bovine PPD may be due to that PPD is prepared from culture filtrate and contain a highly complex mixture of antigens (Fifis et al., 1989) and derived from peripheral proteins with generally weak associations with cell wall and cell proteins (Helenius and Simons, 1975) while *M. bovis* that extracted with lysozymes resulted in the release of water soluble cell wall antigen. Arabinogalactan peptidoglycan complexes of peptides (Adam et al., 1973 and Goren, 1982). Peptidoglycan has adjuvanticity, stimulates macrophage, is mitogenic and has anti-tumor and immunosuppressive activities (Lederer et al., 1975 and Goren, 1982).

In conclusion, filter-sterilized lysozyme extract of *M. bovis* elicited strong cellular responses in *M. bovis* naturally infected cattle. Therefore, this extract may be useful as specific antigen for diagnosis of bovine tuberculosis by means of cellular assay system (skin test) or for measuring humoral immune response using ELISA technique.

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الملخص العـــــربى

تقييم إستخدام أنتيجن " مستخلص الليزوزيم " المحضر من الميكوبكتريا بوفس للكشف عن السل البقرى

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تم تحضير مستخلص الليزوزيم من خلايا الميكوبكتريم بوفس بعد معاملتها بالأسيتون و الإيثير و الكلوروفورم ثم تجفيف هذة الخلايا و تعليقها فى تريس حامض الهيدروكلوريك و تعقيمها بالترشيح بمرشحات الملليبور. تم تقيم هذا المستخلص بمقارنتة بالتيوبركلين الأدمى بإستخدام إختبار الجلد فى الأبقار فى مزارع ماشية بها إصابة وقد تم عزل ميكروب الدرن البقرى منها، كما أخذت عينات سيرم لإجراء إختبار الأليزا بإستخدام كل من المستخلص و ال بى بى دى الأدمى و البقرى. و بالنظر إلى نتائج الأليزا و تحليلها إحصائيا يتضح أن الحساسية كانت(92 , 88 و 84 %) و الخصوصية كانت (85.8 , 85.7 و 71.4 %) و معامل التوقع الإيجابى كان (95.8 , 95.6 و 91.2 %) و معامل التوقع السلبى كان (75 , 66.7 و 55.6 %) و كفاءة التوقع (90.6 , 87.5 و 81.3 %) و أخيرا كان معامل الخطأ (9.4 , 12.5 و 18.7 %) على التوالى لأنتيجن مستخلص الليزوزيم , ال بى بى دى البقرى و ال بى بى دى الأدمى.

و تبين من النتائج تفوق مستخلص الليزوزيم فى الكشف عن السل البقرى سواء عن طريق إختبار الجلد أو سيرولوجيا بإستخدام إختبار الأليزا و ذلك بتقليل التفاعل الغير نوعى للكشف عن السل البقرى فى الحيوانات.

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