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EVALUATION OF *Mycobacterium Bovis* CELL EXTRACT ANTIGENS FOR IN-VITRO DIAGNOSIS OF *Bovine Tuberculosis* IN EXPERIMENTALLY INFECTED GUINEA PIGS

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SUMMARY

M. bovis cell extract antigens as filter-sterilized lysozyme and sarkosyl extracts have been evaluated and successfully used in vitro for diagnosis of bovine tuberculosis.They were compared with the bovine PPD by measuring the cell mediated immunity using the lymphocyte blastogenesis assay (LBA) as detected by tetrazolism (MTT) reduction and skin sensitivity test, as well as measuring the serum antibody levels using ELISA in experimentally infected guinea pigs with *M. bovis* and *M. tuberculosis*. The lymphocytic proliferative response in the infected guinea pig groups 2 weeks post infection was higher than that obtained 4 weeks post infection. The use of lysozyme and sarkosyl extract antigens was found to be advantageous over the bovine PPD in lymphocytic blastogenesis assay due to their abilities to confirm specificity and to discriminate between animals infected with *M. bovis* and those infected with *M. tuberculosis*. The obtained results of the skin sensitivity test revealed that, the bovine PPD, sarkosyl and lysozyme extract antigens were able to differentiate the guinea pigs, infected with typical mycobacteria from those infected with atypical. ELISA results confirmed the superiority of sarkosyl extract over bovine PPD and lysozyme extract in distinguishing between guinea pigs infected with typical and atypical mycobacteria at serum dilution of 1/80 (4,6 and 9 weeks post infection). Both sarkosyl extract and lysozyme extract antigens would be advantageous over bovine PPD in differentiating *M. tuberculosis* infected guinea pigs from *M. bovis* infected group at serum dilution 1/160 while bovine PPD, was not

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able to differentiate between those two groups. The results of ELISA in this work suggested, that *M. bovis* cell extract antigens (lysozyme and sarkosyl extracts) were useful antigens to minimize non-specific reactions in diagnosis of bovine tuberculosis. It was clear from the preliminary results of naturally infected cattle with *M. bovis* that, using the lysozyme and sarkosyl extract antigens could be of value in diagnosis of bovine tuberculosis.

INTRODUCTION

Tuberculosis continues to be a world wide economic and health problem for both human and animals. The human disease is responsible for approximately 3 million deaths annually, while tuberculosis in cattle is a major cause of economic losses and represents a significant cause of zoonotic infection (Pollack and Andersen, 1977). Many national eradication programmes depend on intradermal tuberculin test which is the universal, old and most widely used test for diagnosis of tuberculosis among animals (Pollock et al., 1994). The test however, suffers from insufficient specificity (Pritchard, 1988), as all tuberculins are able to induce reactions in animals sensitized with non-tuberculous mycobacteria. This non-specificity is due to the presence of common antigens in all mycobacteria and most of these antigens are skin-reactive (Daniel and Janicki, 1978). To overcome the problems of tuberculin test, the use of possible alternative laboratory based tests has been investigated (Wood and Rothel, 199[illegible] including a variety of serological methods as e[illegible]zyme linked immunosorbent assay (ELIS[illegible] which has the highest sensitivity and specifici[illegible] than other serological tests for diagnosis of tube[illegible]culosis. Recent advances in instrumentation tech[illegible]nology have provided highly sensitive metho[illegible] with adequate specificity such as the lymphocy[illegible] blastogenesis assay (LBA) for the evaluation o[illegible] the cellular immune response to specific antigen[illegible] and mitogens and to differentiate infections due t[illegible] *M. bovis* and those caused by other mycobacteri[illegible] (Thoen et al., 1980 and Pollock et al., 1994). But due to lack of specific antigen, most of the tests used, were not completely successful and were found to have limitations in terms of sensitivity, specificity or reproducibility. Thus, most of the researches, concerned with tuberculosis were directed towards either the use of highly sensitive immunological assays using different mycobacterial antigens, or the isolation of a purified specific antigen from culture filtrates and cell extracts of mycobacterial antigens, in order to improve the sensitivity and specificity of the test in concern (Auer, 1987 and Fifis et al., 1989). The isolation and purification of individual and specially species-specific mycobacterial antigens to serve as reliable diagnostic reagents has probably been one of the most frustrating in tuberculosis research. A filter-sterilized lysozyme extract is one of the *M. bovis* cell extract antigens prepared for use in skin testing and serodiagnosis of bovine tuberculosis (Hall and Thoen, 1985). Also, a sarkosyl extract

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is one of the *M. bovis* cell extract antigen used in serological diagnosis of bovine tuberculosis. This investigation was designed to prepare a lysozyme extract antigen of *M. bovis* and compare the efficacy of lysozyme extract, sarkosyl extract and purified protein derivative (PPD) of *M. bovis* as antigens in experimental study for diagnosis of *M. bovis* infected guinea pigs.

MATERIAL AND METHODS

MATERIALS: Experimental animals: A total of 32 tuberculin negative reactive albino guinea pigs weighing from 200 to 300 grams were used for the experimental infection with different strains of mycobacteria.

Strains: *M. bovis* AN5 strain, obtained from the *M. tuberculosis* strain C; and *M. intracellulare* standard strain ATCC 13950. All the different mycobacterial strains are standard strains obtained from the Central Veterinary Laboratories Weybridge, England and from Bacteriological Diagnostic Products Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

Antigens: *M. bovis* purified protein derivative antigen: Bovine PPD tuberculin was prepared according to the Central Veterinary Laboratories Weybridge protocol, from *M. bovis* strain AN5. It was available in a concentration of 1 mg/ml and obtained from the Bacteriological Diagnostic Products Department, (VSVRI), Abbasia, Cairo, Egypt.

***M. bovis* sarkosyl extract antigen:** It was previously prepared by Dr. Thoen, Dept. of VMIPM, Iowa State University, Ames Iowa, U.S.A. *M. bovis* filter-sterilized lysozyme extract antigen: It was prepared from *M. bovis* AN5 strain according to Hall and Thoen (1985).

Media: Modified Lowenstein-Jensen (L.J.) medium (Collee et al, 1996). Modified Dorset-Henly synthetic medium (Paterson et al., 1958): These media were mainly used in cultivation of mycobacteria. Nutrient broth medium: (Oxoid); Thioglycollate medium: (Oxoid); Middlebrook 7H9 broth: (Difco.); Roswer Park Memorial Institute (RPMI - 1640) medium: Seromed Biochrom KG; Egg white lysozyme: (Sigma Chemical Co); Phenyl methyl sulfonyl fluoride (C7H7FO2S): Calbiochem - Behring, San. Diego, California, USA.; Phyto-haemagglutinin (PHA - L): Biochrom KG; MTT: [3-(4,5-Dimethyl thiazol-2-Yl) 2,5 diphenyl tetrazolium bromide], Sigma, St. Louis, U.S.A.; Ficoll Hypaque Separating Solution: Biochram KG; Lysing buffer (lauryl sulphate): It composed of 10% SDS in 0.1 N HCl; ELISA coating buffer: 10 mg Carbodiimide; 0.1 M Sodium carbonate and 10 ml Na_2CO_3, (pH 9.6); ELISA wash solution: 0.5 M Sodium chloride and 0.5% Tween 80, (pH 7.6); ELISA diluent: 0.5 M NaCl; 1% bovine serum albumin; 1% Tween 80; ELISA conjugate: Protein A Horse Radish Perox-

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idase (Sigma); ELISA substrate: 25 ml Citric acid; 100 ul Hydrogen peroxide 30% (Merck); 125 ul, ABTS [2,2-azino - di (ethylbenzthia-zolin sulfate)], Sigma.

Samples: Heparinized blood samples: Two ml of blood samples were collected from the experimentally infected and control guinea pigs groups by heart puncture in heparinized syringe to be used in LBA.

Guinea pig serum samples: Serum samples were taken and separated from all guinea pigs one week before infection and four, six and nine weeks later.

Cattle serum samples: Serum samples were obtained from 6 (tuberculin positive) cattle with bacteriologically confirmed infection with *M. bovis* and from 2 non-infected cattle (tuberculin negative) with no recent history of exposure serves as control.

METHODS:

Preparation and standardization of filter-sterilized lysozyme extract antigen:

The method of preparation of lysozyme extract of *M. bovis* was carried out according to Hall and Thoen (1985) using ethyl alcohol-ether-chloroform and lysozyme extraction. The sterility test as well as biological standardization of the antigen were done.

Preparation of *M. bovis* sorkosyl extract: Sarkosyl antigen was previously prepared by Dr. Thoen using 0.6% sarkosyl extracts of heat killed *M. bovis*.

Protein determination: Protein concentration of *M. bovis* filter-sterilized extract and sarkosyl extract antigens was determined according to the method of Groves et al., (1968) using the following formula: Protein concentration (mg/ml) = 1.55 A280 - 0.76 A260. (A is absorbance).

Experimental infection of guinea pigs by mycobacteria: A total of 32 tuberculin negative albino guinea pigs were divided into 4 groups, 8 animals each. One group served as non-infected control and the other 3 groups were inoculated intramuscularly with standard *M. bovis* strain No. AN5, standard *M. tuberculosis* strain (C) and standard *M. intracellulare* strain No. ATCC 13950 respectively. Strains were grown previously on to Lowenstein-Jensen media incubated at 37°C for 4 weeks. The dose of infection was 1 mg/ml for *M. bovis* and *M. tuberculosis* and 10 mg/ml for *M. intracellulare*. The skin test was applied after 4 weeks postinfection and blood samples were collected.

Measurement of cell mediated immune response by lymphocyte blastogenesis assay expressed by MTT reduction: It was applied according to the method adopted by Lucy (1974 & 1977) and modified by Lee (1984). Two ml of

blood sample were collected from each guinea pig (infected with *M. bovis, M. tuberculosis* and from the control non-infected). Mononuclear cells were separated from polymorphonuclear cells based on the density gradient centrifugation using ficoll-hypaque (Boyum, 1968).

Total viable lymphocyte count: The cell number and viability were determined using the trypan blue exclusion test (Mayer et al., 1974).

Setting up of lymphocytes culture: 96 wells flat-bottomed sterile tissue culture plates (Nunc, Denmark) were used for cultivation of mononuclear cells. 100 ul of suspended lymphocytes (0.5 x 106 cells) were inoculated in growth media (RPMI media + 15% FCS). Wells contained growth media plus lymphocytes were treated with either 15 ug/ml PHA mitogen or 10 ug/ml of PPD, or sarkosyl extract or lysozyme extract antigens. In addition, five wells containing growth media only served as medium control. The plates were incubated at 37°C in a humid 5-10% CO2 atmosphere for 72 hours.

Lymphocytes blastogenesis assay (LBA) using MTT reduction (Tada et al., 1986): After cell incubation, MTT was added at 1/10th of the total sample volume (i.e. 10 ul MTT/well). The sample was incubated at 37°C in a 5% CO2 incubator for 4 hours. After incubation 50 ul/well of lysing buffer was added. The plate was incubated overnight for complete cell lysis. The optical density (OD) of each well was determined spectrophotometrically by an automatic plate reader at 570 nm. Mean OD values and least significant difference (LSD) were calculated from six-fold replicates. The significance of the test results was calculated by the F test at $p < 0.05$. When the differences between any two means exceeded the LSD value, they were considered significantly different and vise versa as indicated by Reubel and Bauerfeind, (1989).

Skin reactivity test of guinea pigs: Single intradermal skin tests were conducted on the shaved right and left flanks of each group of guinea pigs (experimentally infected with *M. bovis, M. tuberculosis* or M, intracellulare) using 15 ug/0.1 ml of lysozyme extract, or PPD of *M. bovis* antigens and sarkosyl extract antigen five weeks post infection (Hall and Thoen, 1985). Non-infected guinea pigs (the control group) were also injected intradermally at separate sites with each antigen preparation, buffer controls were included. The injection sites on all guinea pigs were observed 24 hours after injection, and the mean diameter of the response was recorded. The results were statistically analysed using F test (Mann and Whitney, 1974).

Enzyme Linked Immunosorbent Assay (ELISA): (Hall and Thoen, 1985): Microtiter ELISA plates were coated with 50 ul/well of mycobacterial antigens (PPD or lysozyme and or sarkosyl extracts). Antigens were diluted to 10 ug/ml in 0.1

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M sodium carbonate (pH 9.6) with the exception of 4 wells which served as blank wells. Each well then received 50 ul of carbodiimide in 0.1 M sodium carbonate buffer. The plates were incubated overnight at 4°C. The plates were decanted, washed 3 times with PBS, then incubated for 30 minutes at 22°C with 0.01 M ammonium chloride 100 ul/well, except the blank wells, and washed 3 times with ELISA wash solution. Sera were diluted 1:40 in ELISA diluent buffer and 100 ul were added to the first row of wells of the microtiter plates, and serial two fold dilutions of serum were made.. Each plate received negative sera run in the same dilutions to calculate the cut-off value, then incubated at room temperature for 30 minutes on a horizontal shaker. The microtiter plates were decanted, washed 8 times with ELISA wash solution and were allowed to stand inverted for 30 minutes. To each well, 50 ul of protein-A-horseradish conjugated with peroxidase (diluted 1:500) was added and the plates were then incubated for 30 minutes at room temperature. The plates were again washed 8 times with ELISA wash and allowed to stand inverted for 30 minutes. Followed by addition of 150 ul of freshly mixed ABTS to H_2O_2 in 0.05M citric acid (pH 4.0) to each well, the plates were then incubated in the dark for 60 minutes. The optical density (OD) of the color intensity of the reaction was measured at 405 nm using spectra III ELISA reader. A serum dilution was considered positive if it yielded a mean OD of each group equal to/or greater than the cut off value (Dimitri and Mikhail, 1996). Cut off value was calculated according to Nassau et al., (1976) which was equal to the mean OD of negative serum plus 2 standard deviation.

Identification of acid-fast isolates:

Microscopical examination: Smears from suspected colonies were stained with Ziehl-Neelsen. The shape and acid fastness were demonstrated (Koneman et al., 1979).

Bacteriological Findings: Rate of growth: Isolates that grow within 1 month or more were considered slow growers, while less than 7 days were considered rapid growers (Runyon, 1959).

Photochromogenicity (Pigmentation): It was done according to Kent and Kubica (1985).

Growth at different temperature degrees: Slants of Lowenstein Jensen media were incubated at 28°C, 37°C, and 45°C and examined for growth after 7 days.

Niacin test (Kubica, 1973); Nitrate reduction test (Vestal, 1975) and Sensitivity to thiophene-2-carboxylic acid hydrazide (TCH) (Collee et al., 1996).

RESULTS

Results of lymphocyte blastogenesis assay (LBA) using MTT reduction: Lymphocyte blas-

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iogenesis expressed by colorimetric MTT reduction assay using bovine PPD, sarkosyl and lysozyme extract antigens on *M. bovis* and *M. tuberculosis* infected guinea pigs are expressed. Table (1) The highest response was recorded at 2 weeks post infection and began to decrease by the fourth week.

The absolute level of reactivity of PBL in M. bovis infected guinea pigs was 2 weeks post infection.

Results of the skin test reactivity: The results of the skin test reaction obtained with bovine PPD, sarkosyl extract of *M. bovis* and filter-sterilized lysozyme extract antigens are shown in Table 2. It was noticed that, the skin test responses were not observed in the control (non-infected) guinea pigs group when the three mycobacterial antigens were used. Statistical analysis of the results using F test clarified that there is no significant difference ($P > 0.05$) in the skin test reaction in each of the infected groups when tested with the same antigens. Statistical evaluation of the mean skin test diameter recorded that, when using PPD, sarkosyl extract or lysozyme extract, there is a significant difference ($p < 0.05$) between typical and atypical mycobacteria infected guinea pigs.

ELISA results on the infected guinea pigs sera using lysozyme extract, bovine PPD and sarkosyl extract antigens: Table (3) showed the ELISA results on the sera collected from *M. bovis*; *M. tuberculosis* and *M. intracellular* infected using the three different coating mycobacterial antigens. Using lysozyme extract, the serum dilution of 1/80 gave positive ELISA results with typical or atypical mycobacteria infected guinea pigs. When the serum was further diluted to 1/160, ELISA gave negative results with *M. intracellulare* infected guinea pigs. It was demonstrated that the lysozyme extract could differentiate between *M. bovis* and *M. tuberculosis* infected groups at serum dilution 1/160 (4 and 6 weeks post infection), since it gave negative results with *M. tuberculosis* infected guinea pigs. Furthermore, ELISA results were negative with *M. tuberculosis* infected guinea pigs at serum dilution 1/320 (9 weeks post infection) by using lysozyme extract. Concerning bovine PPD, the ELISA could not differentiate between the guinea pigs infected with typical or atypical mycobacteria at serum dilution of 1/80, whereas at serum dilution of 1/160, *M. intracellulare* infected group turned to be negative by ELISA. Bovine PPD could not differentiate between *M. bovis* and *M.tuberculosis* infected groups even at serum dilution 1/320, since it gave positive ELISA reactions with *M. bovis* and *M. tuberculosis* infected groups. Table (3)revealed also that sera of guinea pigs infected with typical mycobacteria could be differentiated from those of atypical mycobacteria by ELISA only using the sarkosyl extract which gave negative results at serum dilution 1/80 with *M. intracellular* infected guinea pigs. Moreover, it was noticed that at serum dilution 1/160, sarkosyl extract could differen-

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tiate between M. bovis and M. tuberculosis infected groups (4 weeks post infection) The sera of guinea pigs infected with M. bovis showed positive ELISA results at serum dilution 1/320 using lysozyme extract, PPD and sarkosyl extract antigen.

Results of ELISA of naturally infected cattle sera: Table (4) indicates that 6 out of 6 cattle sera were positive on ELISA, using lysozyme extract or sarkosyl extract at serum dilution of 1/320, while 3 out of 6 cattle were positive at the same dilutions of serum using bovine PPD. Furthermore, all 6 cattle were tuberculin positive reactors as well as *M. bovis* was isolated and bacteriologically identified from those animals. Regarding the two cattle from the non-infected herd (control negative cattle), the ELISA reactions gave negative results when using the three anti-

Table (1): Results of Lymphocytes blastogenesis assay expressed by OD of MTT reduction using different mycobacterial antigens on *M. bovis* and *M.tuberculosis* infected guinea pigs.

Guinea Pigs Groups Infected with	Mean (OD) of The Reading At							
	2 weeks post infection				4 weeks post infection			
	PPD	Sarkosyl	Lysozyme	L.S.D.	PPD	Sarkosyl	Lysozyme	L.S.D.
M. bovis	0.451	0.523	0.515	0.058	0.291	0.163	0.225	0.054
M. tuberculosis	0.449	0.446	0.505	0.027	0.160	0.121	0.172	0.036
Non-infected Control	0.192	0.083	0.200	0.032	0.140	0.105	0.134	0.027
L.S.D.	0.065	0.043	0.012	-	0.029	0.020	0.031	-

PPD: bovine Purified Protein Derivative; **Sarkosyl:** Sarkosyl extract of ***M. bovis;*** **Lysozyme:** Lysozyme extract of ***M. bovis***; **L.S.D.:** values of least significant difference; **OD:** optical density; **LAB:** Lymphocytes blastogenesis assay.

Table (2): Results of skin test to PPD, sarkosyl extract and lysozyme extract of *M.bovis* antigens in infected guinea pigs with typical and atypical mycobacteria.

Types of antigen	Infected guinea pigs			Non-infected (control) Guinea pigs
	Typical mycobacteria		Atypical myobacteria	
	M. bovis	*M. tuberculosis*	*M. intracellulare*	
Bovine PPD	*6.3 ± 0.83	6.1 ± 0.74	1.12±43	NR
Sarkosyl extract	9.0 ± 0.812	7.6 ± 0.83	0.8± 0.47	NR
Lysozyme extract	8.6 ± 0.44	8.1 ± 0.24	2.4 ± 0.55	NR

1.5 ug of protein / 0.1 ml; * Data expressed as mean diameter of induration (mm) values ± Standard Error (SE); *NR: no response; Results were statistically evaluated using F test.

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gens as well as being tuberculin test was negative.

Identification of reisolated tubercle bacilli from infected guinea pigs: The strain identified as *M. bovis* was slow grower, non-chromogenic, grow at 37°C, negative for the nitrate and niacin tests and sensitive to TCH (inhibition of growth on TCH containing media). *M. tuberculosis* was also identified, which was positive for nitrate and niacin tests and resistant to TCH (presence of growth on TCH containing media).

Table (3): ELISA results using lysozyme extract, PPD and sarkosyl extract antigens at 4,6 and 9 weeks Post *M.bovis* and *M. tuberculosis* infected guinea pigs.

Serum dilutions at 4 weeks PI	*M. bovis lysozyme extract*				Bovine PPD				*M. bovis* sarkosyl extract			
	B.	H.	I.	C.	B.	H.	I.	C.	B.	H.	I.	C.
1/40	+	+	+	-	+	+	+	-	+	+	+	-
1/80	+	+	+	-	+	+	+	-	+	+	-	-
1/160	+	-	-	-	+	+	-	-	+	-	-	-
1/320	+	-	-	-	+	+	-	-	+	-	-	-
Serum dilutions at 6 weeks PI	B.	H.	I.	C.	B.	H.	I.	C.	B.	H.	I.	C.
1/40	+	+	+	-	+	+	+	-	+	+	+	-
1/80	+	+	+	-	+	+	+	-	+	+	-	-
1/160	+	-	-	-	+	+	-	-	+	+	-	-
1/320	+	-	-	-	+	+	-	-	+	-	-	-
Serum dilutions at 9 weeks PI	B.	H.	I.	C.	B.	H.	I.	C.	B.	H.	I.	C.
1/40	+	+	+	-	+	+	+	-	+	+	+	-
1/80	+	+	+	-	+	+	+	-	+	+	-	-
1/160	+	+	-	-	+	+	-	-	+	+	-	-
1/320	+	-	-	-	+	+	-	-	+	-	-	-

B: *M. bovis* infected group; H. *M. tuberculosis* infected group; I: *M. intracellulare* infected group; C: Non-infected control group; PI: post infection

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Table (4). ELISA and Tuberculin results of naturally infected cattle with *M. bovis* using three different Mycobacterial antigens.

Animal Number	ELISA results using												Tuberculin Reactivity
	Lysozyme extract				PPD				Sarkosyl extract				
	1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320	
1	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	-	-	-	-	+	+	+	+	+
3	+	+	+	+	+	-	-	-	+	+	+	+	+
4	+	+	+	+	+	+	-	-	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+
#7	-	-	-	-	-	-	-	-	-	-	-	-	-
#8	-	-	-	-	-	-	-	-	-	-	-	-	-

Control animals (bacteriologically negative cattle to *M. bovis*).

DISCUSSION

Diagnosis of bovine tuberculosis is undertaken by the widely spread tuberculin test. PPD remains the standard preparation for clinical tuberculin testing in both veterinary and human medicine for the diagnosis of tuberculosis (Wood and Rothel, 1994). Despite the application of the intradermal tuberculin test, it has a number of documented problems (Pritchard, 1988). Many studies have been attempted to standardize the dose, route, site of tuberculin injection and the exact time of recording the skin thickness. However, the lack of specificity and sensitivity obligates other evaluation and testing techniques. The non-specific reaction is the most important problem when using PPD tuberculins. 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). During the past half century many researches has been directed towards the isolation of individual antigenic constituents, especially species-specific to serve as a reliable diagnostic reagent, either from cultural filtrate or cell extract (Fifis et al., 1989).

In this study, it was attempted to produce an innovative antigenic extract which is a lysozyme extract of *M. bovis* and a , sarkosyl extract and PPD of *M. bovis* were also studied for diagnosis of bovine tuberculosis in guinea pigs experimentally infected with different mycobacterial strains and under field conditions. These animals can usually

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be diagnosed by the intradermal tuberculin test or by some form of in vitro cellular assay such as lymphocyte blastogenesis assay (Reubel and Bauerfeind), (1989).

For the in-vitro evaluation of proliferation and activation of lymphocytes, the lymphocyte blastogenesis assay (LBA) by the colorimetric MTT reduction was applied. The results in table (1) proved that the use of lysozyme and sarkosyl extract antigens, through the encountered LBA results, would therefore be advantageous over PPD due to their great abilities to confirm specificity and to discriminate between animals infected with *M. bovis* and those infected with *M. tuberculosis* in the early stage of the disease. There was a significant difference between (PBL) stimulation of experimentally infected guinea pigs groups (ranging from 0.523 to 0.446) and non-infected control group (0.200 to 0.083) 2 weeks post infection. Muscoplat et al., (1977) expressed that the infected animal with *M.bovis* exhibited significantly greater stimulation when cultured with PPD than did control animals. In contrast, Thoen et al. (1980) found that there was no significant in vitro lymphocyte responses were observed in the control negative animals. This preliminary results in the guinea pigs experimentally-infected with *M. bovis* and *M. tuberculosis* suggested that lymphocyte immunostimulation tests may be of value in diagnosis of tuberculosis. The present results indicate the superiority of sarkosyl extract and lysozyme extract of *M. bovis* antigens over the bovine PPD in early diagnosis of tuberculosis. Moreover, the cell mediated immune response activity at the second week post infection was higher than the response at the fourth week post infection in two groups of experimentally infected guinea pigs. Regarding to these results Muscoplat et al., (1975) observed a rapid T-cell response at 2 weeks post infection of cattle with *M. bovis*. In comparison of immune responses of *M. bovis* exposed guinea pigs, the results in table (1) indicated that however, the in vitro transformation responses of lymphocytes to bovine PPD was usually lower (0.451) after 2 weeks post infection than the responses to sarkosyl extract antigen (0.523) or lysozyme extract antigen (0.515). Responses which developed at 2 weeks remained relatively higher to those at 4 weeks post infection. It was also noticed that the responses of the peripheral blood lymphocytes (PBL) from non-infected control guinea pigs showed significant lower stimulation to any of the mycobacterial antigens although their response to PHA shows them to be immunocompetent. These results agree with results of Alhaji et al. (1974) who found that lymphocytes from non-infected control animals showed no stimulation by any of the PPD although their response to PHA.

The skin test is one of the oldest immunological tests still in widespread use for, the diagnosis of tuberculosis among animals (Pollock et al., 1994). It was used to detect specific cellular immunity. Regarding the skin test of infected and

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non-infected guinea pigs with different mycobacterial strains using PPD, sarkosyl extract and lysozyme extract antigens, the results depicted in table (2) revealed that the three antigens could be able to differentiate between the infected and non-infected (control) groups. These results proved that both lysozyme and sarkosyl extract of M. bovis antigens could stimulate delayed type hypersensitivity (DTH) reaction similar to the bovine PPD. Thus these findings agree with the results reached by Hall and Thoen (1983) who found that a filter-sterilized lysozyme extract of *M. bovis* was a potent skin test antigen in sensitized guinea pigs. Results of the skin reactivity indicated that, sarkosyl extract gave the greatest response (9.0) with M. bovis infected group, followed by lysozyme extract (8.6) and then bovine PPD (6.3) and this which may be due to the components mediated the DTH responses in PPD may be different from those in a filter-sterilized lysozyme extract (Hall and Thoen, 1985). Moreover, Daniel and Janiki (1978) found that the lack of the sensitivity of bovine PPD may be due to that it was a crude mixture of a highly variable composition. Janicki et al., (1971); Turcotte and Boulanger (1971) and Daniel (1980) found also that PPD is a non-uniform product which varies considerably in composition and activity. However the potency of a sarkosyl and a lysozyme extract antigens were non-significantly greater than that of bovine PPD by statistical analysis using F test. Also by using F test there was no significant difference in skin response in each of the infected group when tested with the same antigens. Statistical analysis of the results of the skin test disclosed the capability of the lysozyme extract, sarkosyl extract and bovine PPD antigens to distinguish between groups infected with typical mycobacteria (*M. bovis* and *M. tuberculosis*) and those with atypical mycobacteria (*M. intracellulare*). This agrees with results of Chaparas and Malomey (1978), who found that the homlogous reaction was higher than the heterologous one.

ELISA was applied as a sensitive method for measurement of antibodies in sera of tuberculous animals (Engvall and Perlmann, 1972). The detection of anti-mycobacterial antibodies in the sera of infected and non-infected guinea pigs with different mycobacterial strains, were evaluated by ELISA using lysozyme extract, bovine PPD and sarkosyl extract as coating antigens. The obtained results are displayed in table (3). This revealed that the sarkosyl extract is highly specific, hence its ability to differentiate between the serum of guinea pigs infected with typical mycobacteria from those infected with atypical mycobacteria at serum dilution of 1/80. These findings are supported by the results recorded by Mikhail et al. (1997) who reported that the sarkosyl antigen was sensitive and specific when used for the diagnosis of bovine tuberculosis by the ELISA technique. Sarkosyl extract succeeded in differentiating between *M. bovis* infected guinea pigs and *M. tuberculosis* infected group at serum dilution of 1/160 four weeks post infection and at serum dilution of

1/320 at 6 and 9 weeks post infection. These observations agreed with those of Hammam and Barsoum (1994) and Mikhail et al. (1977) who reported that sarkosyl extract was more specific than bovine PPD.

Prominent attention must be given to the lysozyme extract which gave negative ELISA reaction at serum dilution 1/160 with serum obtained from *M. tuberculosis* infected group at 4 and 6 weeks post infection and at 1/320, 9 weeks post infection. The lysozyme extract of *M. bovis* can be able to differentiate *M. bovis* infected group from *M. tuberculosis* infected group, while bovine PPD could not differentiate between *M. bovis* and *M. tuberculosis* infected groups of guinea pigs even at serum dilution of 1/320.

The failure of bovine PPD antigen to differentiate between serum of infected guinea pigs with typical mycobacteria from those infected with atypical mycobacteria at serum dilution of 1/80 could be described by the fact that bovine PPD has many individual and common antigens (Stavri et al., 1982). These common sharing antigens are prevailing among many species in the genus Mycobacterium as recorded by Benjamin et al (1984). Also, Bennedsen (1966) and Lepper and Pearson (1975) reported that at least there are two surface antigens giving rise to antibody formation one of which is specific while the other is shared with a number of mycobacterial species. The higher ELISA values obtained after using the sarkosyl extract when the bovine PPD was used may be due to the fact that PPD is prepared from a cultured filtrate which contains a highly complex mixture of antigens (Fifis et al., 1989) while sarkosyl extract was prepared from cells by sarkosyl, which is an ionic detergent which facilitated the release of solubilized integral proteins tightly bound to the cell wall and cell membrane (Hall and Thoen 1983).

Encouraging results were obtained by using lysozyme and sarkosyl extract antigens in ELISA under field condition, on a bacteriologically positive *M. bovis* cattle sera. The result of ELISA declared that bovine PPD was of lower sensitivity in comparison with lysozyme extract. This result comes in accordance with Hall and Thoen (1985). Also the PPD was less sensitive than the sarkosyl extract which agrees with Mikhail et al. (1997) who found that sarkosyl extract was more sensitive than bovine PPD when ELISA was used on the sera of *M. bovis* infected cattle.

In conclusion, lysozyme and sarkosyl antigenic extracts of *M. bovis* elicited strong cellular responses in *M. bovis-infected* guinea pigs. They have the ability to differentiate between typical and atypical mycobacteria infected groups on the basis of cellular and humoral immune response. Therefore, these proteins may be useful as specific antigens for diagnosis of bovine tuberculosis by means of cellular assay systems, and skin test or for measuring humoral immune response using

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ELISA. It is recommended that further studies on the immunological characteristics of *M. bovis* antigenic extracts are needed to improve the efficiency of serological diagnosis of mycobacteriosis and to evaluate their specificity and sensitivity on a large scale. Further studies are also needed to evaluate lysozyme and sarkosyl extracts of *M. bovis* as skin test antigens on cattle in herds in which *M. bovis* infection has been diagnosed.

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