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EVALUATION OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) AND PATHOLOGICAL FINDINGS IN THE DIAGNOSIS OF TUBERCULOSIS IN CAMELS (*CAMELUS DROMEDARIUS*) IN EGYPT

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SUMMARY

A modified ELISA for serological evaluation of new standard key instead of the present key for interpretation of single intradermal tuberculin test in camels were conducted on sera taken from field animal and experimentally infected camels with a virulent Mycobacterium bovis.

The results showed a high specificity of newly standard key than the present key for the detection of anti-tuberculous antibodies in sero-diagnosis of tuberculosis in camels. Moreover, a good correlation between post mortem findings, bacteriological isolation and the ELISA test results was found.

Histopathologically, tuberculous lesions in experimentally infected camels were characterized by a productive and proliferative response of fibrous tissue with no exudative reaction and a few Langhan's giant cells. This is in contrast to lesions observed in domestic ruminants. The main feature of the lesion is caseous necrosis with some calcification and few giant cells. Acid-fast bacteria (AFB) are rarely evident.

INTRODUCTION

Unlike in other species of animals, serological diagnosis of tuberculosis among camels has received little attention. However, (Abo-Zeid and

Hammam 1993) reported a good correlation between a haemagglutination test and tuberculin tests, but they concluded that the serological test needs more investigations to be used for diagnosis of tuberculosis in camels. Enzyme Linked Immunosorbent Assay (ELISA) was recommended by many authors (Ritacco et al., 1987; Casillas et al., 1995 and Lilenbaum, 1999) as a highly sensitive and specific serological test, as a complementary method to tuberculin test especially for anergic tuberculous cattle and for collective diagnosis to elucidate clinical suspicious of disease or in the first steps of control programs for identification of foci among cattle. In relation to camels, there is scanty information available in literature concerning the use of ELISA or pathological changes for the diagnosis of tuberculosis (Bush et al 1990).

The purpose of this study was to use ELISA purified protein derivative (PPD) assay for serological evaluation of a newly standard interpretation key recommended by (Selim et al. 2002) for interpreting cervical skin intradermal tuberculin test in camels which considered a positive reactor which gave more than 7 mm and 6.5 mm difference in skin fold thickness with human and bovine PPD tuberculin, respectively, instead of the currently used tuberculin test which consider the positive test that gave more than 4 mm with both types of tuberculinís. In addition, to determine the specific and characteristic gross and histopathological lesions of tuberculosis of bovine type infection in lymph node and other tissues or internal organs of slaughtered experimentally infected camels.

MATERIAL AND METHODS

Sera:

A total of 111 serum samples were obtained from 107 camels in abattoirs and from 4 experimentally infected camels with M. bovis in the Faculty of Veterinary Medicine, Cairo University.

Antigens:

Bovine purified protein derivative (PPD) supplied from the Veterinary Serum and Vaccine Research Institute (VSVRI), Cairo, Egypt was used for coating the ELISA plates, in addition to human PPD tuberculin used with bovine PPD tuberculin for cervical intradermal tuberculin test in camels.

ELISA test procedure:

ELISA was performed as described previously by (Narayanan et al., 1983 and Ritacco et al., 1990) with some modification. Flat bottom microtitre plates (Dynatech) were coated with 50ul (10ug/ml) of bovine PPD antigen diluted in carbonate buffer (pH 9.6) and incubated for 20 hours at 4oC in a humidified atmosphere. After a single wash with PBS-Tween 20 (0.05% w/v) (PBST), wells were blocked with 200ul of dried milk diluted 1% w/v in PBST (PBSTM) and incubated for 1 hour at 37oC. The PBSTM was then tipped off and the plates patted dry. Serum samples to be tested for antibody content were diluted 1:50 and added to 100ul/well and incubated at 37°C for 60 minutes.

After 3 washings with phosphate buffered saline (PBS) (pH 7.4) containing 0.01% tween 20. 150ul of protein "A" horseradish peroxidase conjugate (1:1000) (PA-HRPO) was added to each well and the plates were then incubated at 37oC for 60 minutes. The plates were then washed again three times followed by the incubation of substrate at 37oC for 15 minutes. A working solution of substrate was prepared using H2O2 and 2-2 azino-di (3 ethyl benthiosaline-6-sulphonate, ABTS) in citric acid. It was prepared by dissolving ABTS-5 mg in 10 ml citric acid buffer and 3ul 30% H2O2. The optical density recorded at 405 nm in a Dynatech micro-ELISA auto reader monitored the reaction. An ELISA reading that is equal to or higher than double fold of the ELISA reading of the negative control is considered as positive (Bassiri et al., 1993).

Sensitivity and specificity of the present and new recommended standard keys for interpretation of skin reactions in camels in comparison to that of ELISA were calculated according to the following equations recommended by (Francis et al., 1978) using Bacteriological examination as a key test.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}} \times 100$$

Histopathological investigation of experimental infected camels:

From four experimentally infected camels with M. bovis which slaughtered after 18 months post infection, tissues specimens were collected from lymph nodes and internal organs showing tuberculous lesion and fixed in 10% buffered formalin and processed for H & E staining (bush et al 1990).

Post-mortem and bacteriological examination:

All animals were examined carefully to detect any post-mortem changes including serial incision of all lymph nodes of animals.

Direct smears were made from lesions for staining with Ziehl-Neelsen stain and samples were obtained for cultivations and performed by the standard procedures (Roberts et al., 1991).

Films were made from all cultures and stained with Ziehl-Neelsen and Gram stain. Identification of isolates was carried out according to Chadwick (1981).

RESULTS

The test results of ELISA of 107 camels using bovine PPD type are summarized in Table (1). Nine camels were reported as ELISA positive. Gross lesions (tuberculous like lesion in necropsied ani-

mal) could be detected in 12 camels only. Bacteriological examination, revealed the isolation of 2 *M. bovis*, one *M. africanum*, 5 isolates of coryne pseudotuberculosis, one of atypical mycobacteria mixed with coryne pseudotuberculosis, one atypical mycobacteria could be isolated and no isolates could be detected from two camels. At the same time, no bacteria could be isolated from the rest of tuberculin positive camels.

Comparison of the present and new recommended standard keys for assaying tuberculin tests in camels is shown in Table (2). Using the currently used standard key to evaluate skin reactions. 31 animals could be considered as positive skin reactors with human PPD while versus to only 18 animals were positive by using bovine PPD out of the 107 animals tested were reacted. Evaluation skin reactions based on the new recommended standard, revealed 8 positive reactors out of 107 camels with human tuberculin while with bovine PPD tuberculin 4 animals reacted positively.

Table 1: Correlation between the tuberculin reaction, ELISA and bacteriological examination of 12 camels showing visible lesions.

Serial No.	Difference in skin fold thickness with		ELISA* Results	Bacteriological Examination
	Human PPD tuberculin	Bovine PPD tuberculin		
1	5.6	3.4	- Ve	*C. Pseudotuberculosis*
2	5.9	4.5	- Ve	*C. Pseudotuberculosis*
3	6.0	4.7	+ Ve	*C. Pseudotuberculosis*
4	6.2	4.9	+ Ve	*C. Pseudotuberculosis*
5	7.6	5.7	+Ve	*C. Pseudotuberculosis*
6	8.6	7.2	+Ve	-ve isolates
7	8.4	5.3	+Ve	MOTT
8	8.9	6.0	-Ve	-ve isolates
9	9.1	6.0	+Ve	MOTT +C *pseudotuberculosis*
10	9.8	7.0	+Ve	*M. Africanum*
11	13.0	7.9	+Ve	*M. Bovis*
12	14.3	9.8	+Ve	*M. Bovis*

* Coating antigen is bovine PPD

Table 2: Results of tuberculin skin test in 107 camels tested by luman and bovine tuberculin and interpreted by the present and new standard keys.

Total Camel Exam.	* Current standard				* New standard			
	Human PPD		Bovine PPD		Human PPD		Bovine PPD	
	No.	%	No.	%	No.	%	No.	%
107	31	28.9	18	16.8	8	7.5	4	3.7

* Current standard positive = 4 mm or more difference in skin fold thickness.
** New standard positive = more than 7 mm in mammalian PPD and 6.5 in bovine PPD difference in skin fold thickness.

Table 3: Comparison between sensitive and specificity percent of skin reactions with mammalian and bovine PPD interpreted by present and new recommended standard keys of interpretation with ELISA.

	Present standard		New standard		ELISA
	Mammalian PPD (7mm or more)	Bovine PPD (6.5mm or more)	Mammalian PPD (7mm or more)	Bovine PPD (6.5mm or more)	
Sensitivity	100	100	100	100	100
Specificity	73.1	85.6	95.2	99	94.2

The sensitivity and specificity of ELISA in the present and new recommended standard keys in Table (3) which depending upon the data shown in Table (1). The sensitivity of mammalian and bovine PPD either interpreted by two types of interpretation was 100 %, but specificity of skin reactions with mammalian PPD interpreted with the present and new standard key was 73.1 and 95.2 %, respectively, while with bovine PPD was 85.6 and 99 %, respectively. In ELISA, specificity was 94.2 %.

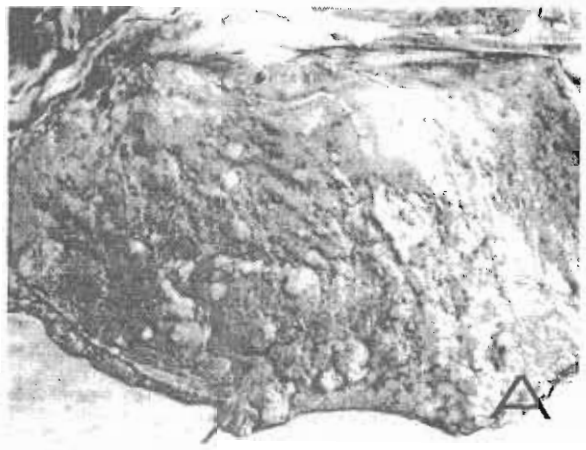

Fig. (1A): Lung showing numerous tubercles of varying size and age scattered on the surface.

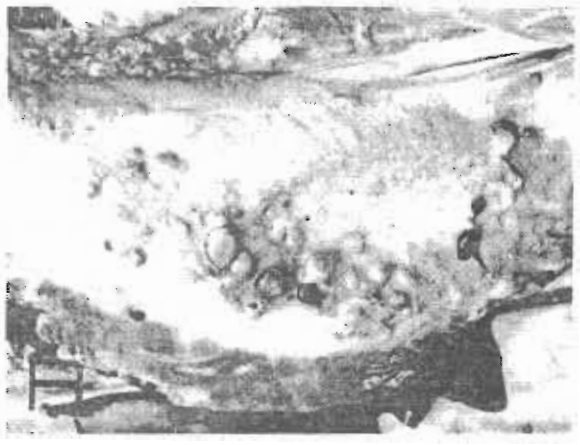

Fig. (1B): Diaphragmatic pleura showing numerous raised plaques and grape like nodules.

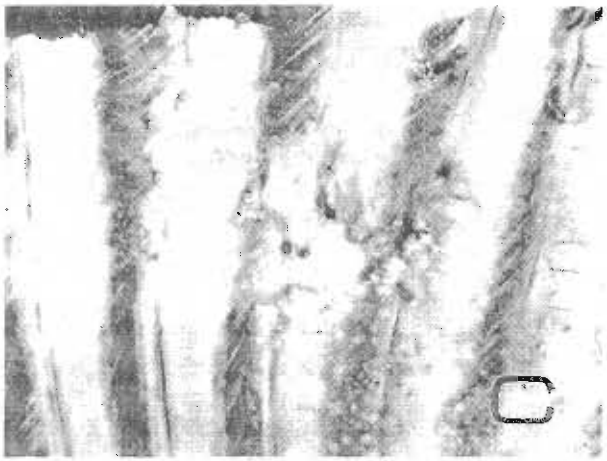

Fig. (1C): Discrete miliary tubercles attached to the pleura of the thoracic wall.

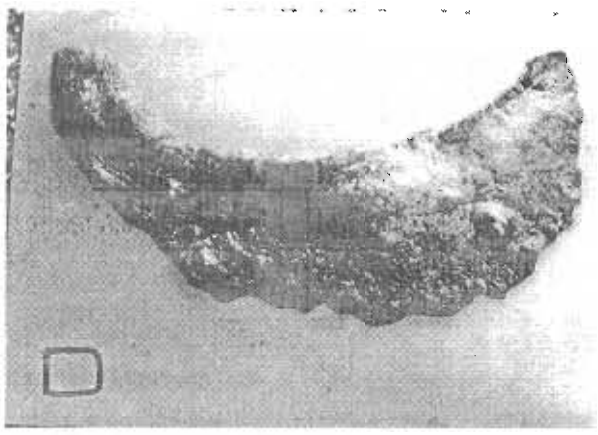

Fig. (1D) Spleen showing prominent caseous spheroidal masses of varying size.

Fig. (1E): Liver showing caseated nodules

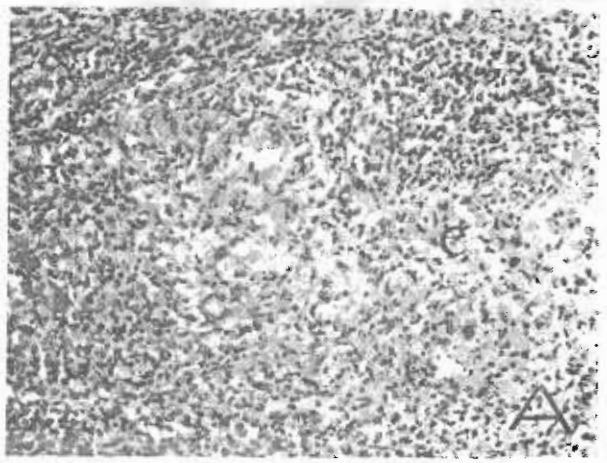

Fig. (2A): Lung showing an early tuberculous reaction consisting of epithelioid cells (e) surrounded by lymphocytes (H & E x33).

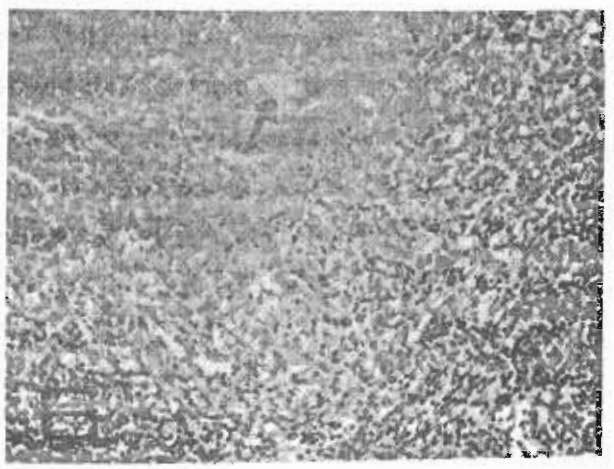

Fig. (2B): Progressing tuberculous nodule showing central cassation (H & E x 33).

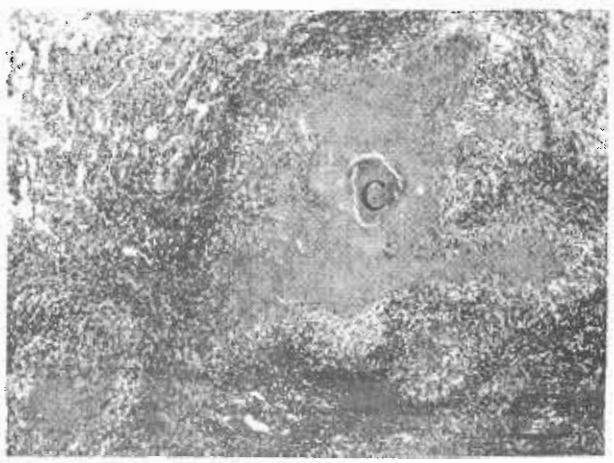

Fig. (2C): An old tubercle nodule in which cassation extended to involve large area, become calcified (c) and showed a peripheral intense lymphocytic cell infiltration (H & E x13.2).

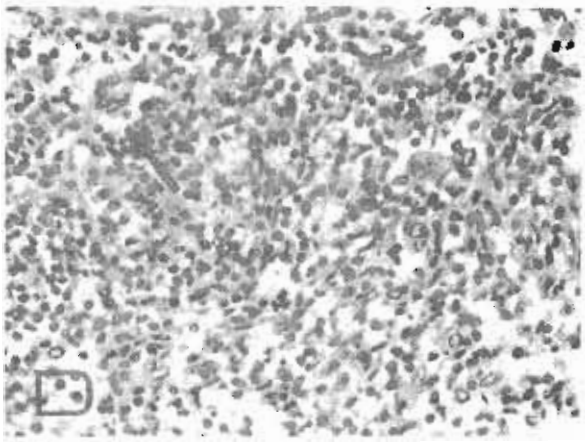

Fig. (2D): Margin of an old tubercle showing a single giant cell (arrow), mononuclear cells mainly lymphocytes, intact epithelioid cells and fibrocytes (H & E x66).

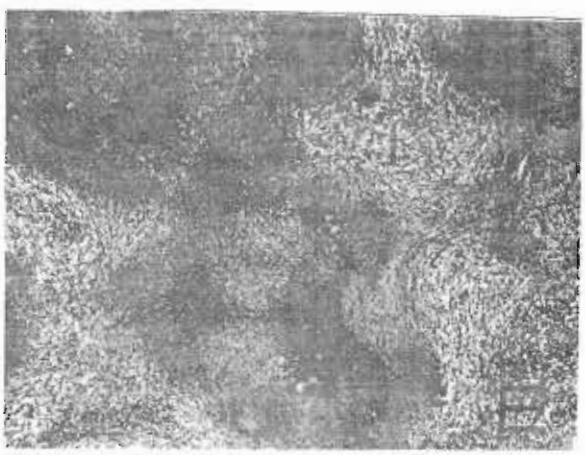

Fig. (2E): Spleen showing prominent fibrous reaction (f) surrounding a central core of cassation and calcification (H & E x13.2).

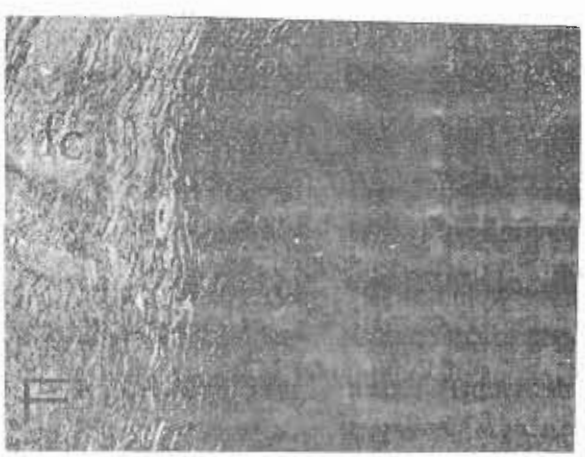

Fig. (2F) Pleural grape like nodule showing diffuse cassation necrosis, central calcification and a peripheral thick dense fibrous capsule (fc) showed scantly lymphocytes (H & E, x 132).

Pathological findings of experimentally infected camels with M. bovis:

Grossly, numerous tuberculous nodules of varying sizes and types were mostly and commonly affecting, the lungs, pleura, pericardium bronchial and mediastinal lymph nodes in addition to liver, kidneys and spleen. The lungs showed numerous miliary tubercles of varying size and age widely scattered on the surface and deep in the lung parenchyma. In addition, larger nodules up to 5 cm diameter and even large areas of the lung tissue appeared solid and caseated (Fig. IA). The pleura showed numerous raised plaques and masses of grape-like nodules attached to the diaphragmatic pleura and that of the chest wall. Discrete dark red soft nodules were also observed (Fig. 1B and C). The spleen showed prominent caseous spheroidal masses of varying size from 0.5 cm to a hazel nut (Fig. 1D). The liver was focally involved with the formation of large caseated nodules (Fig. 1E). Similar lesions were also observed in the kidneys but were smaller and sporadic.

Microscopically examination Generally, typical histological characteristics of tuberculous lesions with the difference that Langhans giant cells were very rare and there was an increasing tendency for fibrous tissues proliferation. Early lesions started as aggregation of epithelioid cells surrounded by a large number of lymphocytes (e in Fig. 2A). This was followed by central caseation (Fig. 2B), which extended peripherally to involve larger areas, became calcified and showed a peripheral intense lymphocytic cell infiltration and fibrous connective tissue reaction (C in Fig. 2C). Scantly Langhans giant cells were seen in the marginal zone of old tubercles (Fig. 2D). The spleen characteristically showed an excessive loose fibrous connective tissue reaction surrounding a central core of cassation and calcification (f in Fig. 2E). Pleural nodules generally showed a wide caseocalcified center encircled with a thick dense fibrous capsule, which showed scantly mononuclear cell infiltration and no giant cells (FC in Fig. 2F). The dark red diaphragm nodules observed grossly were mostly hemorrhagic and showed no characteristic tuberculous changes.

DISCUSSION

The ELISA-PPD described herein, using protein A labeled with horseradish peroxidase conjugate as a substitute for the specific anti-camel IgG conjugate, can be applied in the detection of antituberculosis antibodies in dromedary camels. Investigation of 107 serum samples of blindly selected camels tested by ELISA could detect 9 cases as positives with 100% sensitivity but by comparison with bacteriological findings the specificity of the test was low as the true positive were only 3 camels from which M. bovis and M. Africanum were isolated, but the test could pick

up 7 cases as positives where Coryne Pseudotuberculosis and MOTT were isolated. These results of low isolation of M. bovis demonstrate that the skin reactions of the necropsies positive reactor by present interpretation key can be attributed to the non-specific sensitization of these animals and this; using the new recommended interpretation of tuberculin test to increase the specificity of skin test can solve problem. The isolation of M. bovis from suspected tuberculous lesions of slaughtered camels in the present investigation coincide with the result of many investigators (Abo-Zaid and Hammam, 1993; Mikhail, 1985 and El-Sergany et al., 1990) who failed to isolate Mycobacterium from Tuberculosis like lesions. But, the isolated organisms were coryne bacterium, staphylococci, streptococci, citrobacter species and E. coli. For confirmation of ELISA results a comparison was carried out with the cervical skin reactions by using mammalian and bovine tuberculinís on the same animals but interpretation of the degree of increase in skin thickness was evaluated by two systems of standardization. Results in Table (3) showed that specificity of mammalian PPD was 73.1% only and increased to 95.2% by using the new standard key and similar results were observed with bovine PPD in which the specificity percent increased from 85.6% to 99%. These results confirm those obtained in a previous study (Selim et al., 1997), which recommended a new standard key for the diagnosis of tuberculosis in camels. The percent of specificity in ELISA (94.2 %) was nearer to the percents of tuberculin tests evaluated by the newly standard keys.

The same correlation has been reported during investigation in camels (Bush et al, 1990). The high specificity of ELISA (94.2%) and sensitivity (100%) encourages us to recommend ELISA as a serological test for screening of dromedary camels during the programs of controlling tuberculosis and positive animals must be tested with a tuberculin test using bovine PPD and skin readings must be evaluated with the new recommended standard key. In the first step of a control program, a method with good sensitivity is required and a little reduction in specificity is acceptable (Lilenbaum et al., 1999). As diagnosis of tuberculosis in slaughtered animals depends mainly on post mortem and histopathological investigations and since information is very scanty in literature, a full description of the gross and microscopic tuberculous lesions in tissues collected from experimentally infected camels with M. bovis was made. It was observed that histopathological features of tuberculous lesions in camels had a productive proliferative response of fibrous tissue with no exudative reaction and a few Langhanís giant cells. The same histopathological findings coincide with those described in bacterian camels (Casilas et al., 1995).

These histopathological features are in contrast to lesions observed in cattle where the centre has a caseous necrosis, usually with some calcification with a boundary of epithelioid cells and some of which form multinucleated giant cells and few to numerous lymphocytes and neutrophils. An outer border of fibrous connective tissue is usually present, giving the lesion a focal appearance and providing encapsulation to some extent (Thoen and steel, 1995).

In conclusion, the results of the present investigation necessitate the application of newly standard interpretation key for single intradermal tuberculin by using bovine tuberculin under the Egyptian conditions in addition to adopting serological tests such as ELISA due to high sensitivity and specificity in order to confirm diagnosis of bovine tuberculosis.

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