EVALUATION OF MYCOBACTERIUM BOVIS SARKOSYL EXTRACT FOR DIAGNOSIS OF BOVINE TUBERCULOSIS IN BUFFALOES

By

Nasr, E.A.*; Mikhail, D.G.*; Hussein, A. Z.* and Hammam, H.M.**

* *Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.*
** *Central Laboratory for Control of Veterinary Biologics, Abbasia, Cairo.*

ABSTRACT

A modified indirect ELISA was conducted on sera from 50 tuberculin positive and 38 tuberculin negative buffaloes, using human and bovine PPD tuberculins in addition to sarkosyl extract from Mycobacterium bovis. The results revealed that the sarkosyl extract gave the best discrimination between tuberculous and non-tuberculous animals, as higher sensitivity, specificity and efficiency of prediction than either types of PPD was recorded. The probability of misclassification cleared that only 18% of tested animals would be misclassified by the sarkosyl extract, in comparison to 28% and 25.7% for human and bovine PPDs respectively. The use of serological assays, using semi purified antigens, such as sarkosyl extract, as a complement to intradermal tuberculin skin test, would greatly increase the efficiency of eradication programs.

INTRODUCTION

Mycobacterium bovis, the causative agent of bovine tuberculosis, can infect not only cattle, but also a wide range of mammalian species including humans. (**Moda *et al.*, 1996**). In developed countries, however, its major importance is the economic impact on animal industries, as it requires massive annual expenditure on control and eradication programs. (**Krebs *et al.*, 1997**).

Despite the widespread use of the intradermal tuberculin test, it does have a number of well-defined problems (**Pritchard, 1988**). The problem of false-positive reactions due to antigenic cross-reactivity with atypical mycobacteria is the most important.

The problems encountered in tuberculin test lead to many attempts to develop alternative tests of which ELISA gave promising results, however,

the antigens used, suffered also from lack of specificity (**Daniel and Debanne, 1987**).

In the present investigation, a soluble sodium lauroyl sarcosinate (Sarkosyl) extract of virulent *M. bovis*, in addition to human and bovine purified protein derivative (PPD) tuberculins, were used as antigens in ELISA, to test its efficiency in detecting anti-mycobacterial antibodies in sera of buffaloes naturally infected with *M. bovis*.

MATERIAL AND METHODS

Serum samples:

Serum samples were collected from two groups of buffaloes:

Group (1): Fifty serum samples from buffaloes designated as tuberculin test reactors, after being tested by the single intradermal tuberculin test, using human PPD tuberculin.

Group (2): Thirty-eight serum samples from tuberculin test negative buffaloes were further classified into two subgroups:

A. Twenty-two serum samples from farms with history of bovine tuberculosis (contact).
B. Sixteen samples from tuberculin negative buffaloes from farms with no history of tuberculosis. All samples were collected during the period from March 2000 to April 2001.

Antigens:

1. Sarkosyl extract:

It was kindly supplied by **Dr. C.O. Thoen**, Iowa State University, USA. Briefly, cells suspension of heat-killed *M. bovis* was extracted with 0.6% sodium lauroyl sarcosinate (ionic detergent) in phosphate buffer saline pH 7.2 at 4°C overnight, then ultra centrifuged and the supernatant was concentrated by negative pressure. The protein content of the concentrate was determined by the method of **Lowry *et al.*, (1951).**

2. Human and bovine PPD tuberculins:

It was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

ELISA:

ELISA was carriedout as described by **Hanna *et al.*, (1989),** using flat-bottomed microtiter plates (Dynatech Immulon II), protein G horseradish peroxidase conjugate, Ortho Pheneline Diamine (OPD) substrate and 2.5 M sulfuric acid as stopping solution. The concentration of the antigens was 1 ug per well for sarkosyl extract and 1 mg per well for both types of PPD. The optical density was recorded using ELISA reader at 492 nm. A reaction was considered positive if it gave optical density (OD) equal to or greater than the

mean OD of the negative control plus two standard deviations (**Tandon *et al.*, 1980**).

Data analysis:

- Sensitivity, specificity, efficiency of prediction and error of prediction were estimated according to **Daniel and Debanne, (1987)**.
- The probability of misclassification (Pm) was calculated according to **Sheldrake *et al.*, (1983)** as follows :

For each antigen, five cutoff settings were chosen. At each setting, the number of tuberculous buffaloes, the test result of which was below the cutoff setting (false negative prediction) was divided by the total number of tuberculous buffaloes. Similarly, the number of buffaloes with no visible lesions (NVL), the test result of which was above the cutoff setting(false positive prediction) was divided by the total number of NVL buffaloes.

These statistics were plotted, the point at which the two curves crossed represent the point at which the proportion of false positive prediction equaled the proportion of false negative prediction.

RESULTS AND DISCUSSION

Most national programs for eradication of bovine tuberculosis rely upon the detection of cellular immune response using the tuberculin test, as the delayed type hypersensitivity is indicative of infection or exposure to mycobacteria. While the detection of anti-mycobacterial antibodies is more closely related to extent of bacterial multiplication and antigenic load in infected animals (**Harboe *et al.*, 1990**), the use of serological assays as a complement to tuberculin test is important to increase the efficiency of eradication programs.

It is clear from Table (1) that out of 50 buffaloes reacted positively to human PPD tuberculin skin test, only 42 were positive to the bovine PPD tuberculin test. **O'Reilly, (1992)** stated that human PPD appear to be less potent and less specific than bovine PPD. Also **O'Reilly and MacClancy, (1975)** mentioned that bovine PPD gave better discrimination between tuberculous and non-tuberculous animals than human PPD, and **Lepper *et al.*, (1977 a)** found that the specificity of bovine PPD was superior to that of HCSM tuberculin.

This higher specificity of bovine PPD is supported by the fact that the 8 buffaloes which were negative to bovine PPD, showed no visible lesions and no *M. bovis* was isolated.

Out of the 42 bovine PPD-positive animals, 32 showed visible lesions, out of which M. bovis was isolated from 20 cases. The 10 NVL animals may be in an early stage of infection. Such conclusion is supported by that M.

bovis was isolated from 2 cases. **Plackett *et al.*, (1989)** suggested that some animals, with low level of infection, may fail to yield M. bovis on culture.

As regards to tuberculin negative animals from tuberculous herds, only two cases were found to be tuberculous and M. bovis was isolated from them (anergic). These two animals were slaughtered as they were positive for brucellosis. **Lepper *et al.*, (1977 b)** stated that anergy may be due to a very early stage of infection, healing of lesions in advanced cases or other factors such as stress or malnutrition, however the role played by brucella infection needs to be investigated.

As regards to tuberculin negative buffaloes from herds with no history of tuberculosis, the results of tuberculin test coincided with the Post mortum findings and isolation. This indicates that, in case of free herds or very low prevalence, the tuberculin skin test showed high specificity and could be relied upon.

It evident from Table (2) that, in animals with visible lesions, the sarkosyl extract detected more positive cases than either types of PPDs .The same finding was observed in case of NVL animals.

Concerning contact animals (group 2A), 2 buffaloes with visible lesions were correctly classified as positive by the sarkosyl extract, while only one case was detected by both human and bovine PPDs antigens. Also, the sarkosyl extract showed higher specificity in tuberculin negative buffaloes (group 2B).

The purified protein derivative (PPD) tuberculin contains a complex mixture of proteins present in culture filtrate, some of which possess some species specificity, while others showed antigenic cross reactivity that lead to false positive results in both tuberculin test and serological assays.

Many physico-chemical approaches have been used to obtain more purified mycobacterial antigens with varying degrees of success. One of these approaches is the use of anionic detergents. The PPD contains peripheral proteins with generally weak associations with the cell wall and cell membrane and/or proteins released into the medium during growth. The integral proteins are tightly bound to cell wall and cell membrane and are solubilized by detergents {**Helenius and Simons, (1975) and Hall and Thoen, (1983)**}. This may explain the higher sensitivity of sarkosyl extract than both types of PPD's. **Thoen *et al.*, (1992)** recorded high sensitivity with sarkosyl extract, in ELISA, for diagnosis of bovine tuberculosis in Elk. Also **Hall and Thoen, (1986)** using another ionic detergent, sodium deoxcholate (DOC) and stated that there was a significant difference between DOC extract and bovine PPD in ELISA.

The higher specificity of sarkosyl extract may be explained by the statement of **Daniel and Debanne, (1987)** that specificity in ELISA is greatly affected by the antigen used and that the use of semipurified antigen lead to higher specificity. Also, **Daniel, (1988)** mentioned that the serodiagnostic test

characteristics depend significantly upon the antigen used, and the most favourable test characteristics have been achieved using antigens that are substantially purified.

Although the sarkosyl extract and bovine PPD were prepared from *M. bovis*, it seems that the sarkosyl extract contains antigenic constituents that is able of detecting specifically anti -mycobacterial antibodies, while bovine PPD is more suitable for detection of in vivo delayed type hypersensitivity.

Concerning the comparison between the three antigens to predict infection status correctly, it is evident that the sarkosyl extract has the lowest probability of misclassification (18.2 %), while human and bovine PPD's showed higher probability of misclassification (28 % and 25.7 %, respectively). This means that, testing a herd with sarkosyl extract, only 18% of the animals will be misclassified, whether as false positive or false negative, while in case of human and bovine PPD's, 28% and 25.7% of the tested animals will be misclassified.

It is recommended to use a serological assay, which is more closely related to bacterial multiplication and antigen load, as a complement to tuberculin skin test. The use of semi purified protein antigens may result in more favorable test characteristics and better discrimination between tuberculous and non tuberculous animals.

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Table (1): Results of bovine PPD tuberculin test, PM findings and mycobacteriological examination of human PPD tuberculin positive buffaloes.

Group	Number	B-PPD tuberculin test		PM findings		Isolation of M. bovis	
		+	-	+	-	+	-
Tuberculin positive	50	42	8	32	18	20	12
Tuberculin negative (contact)	22	0	22	2	20	2	20
Tuberculin negative	16	0	16	0	16	0	16

Table (2): Results of ELISA on sera from tuberculin positive and negative reactors using three different antigens.

Group	PM findings	Sarkosyl		Bovine PPD		Human PPD	
		+	-	+	-	+	-
Group (1) tuberculin positive	VL(32)	30	2	27	5	24	8
	NVL(18)	12	6	10	8	8	10
GROUP (2A) tuberculin negative(contact)	VL(2)	2	0	1	1	1	1
	NVL(20)	1	19	9	11	10	10
Group (2B) tuberculin negative (neg. herd)	VL(0)	0	0	0	0	0	0
	NVL(16)	0	16	3	13	6	10

VL = Visible Lesions

NVL= Non-visible Lesions

Table (3): Diagnostic test characteristics of ELISA using three different antigens.

	Sarkosyl	Bovine PPD	Human PPD
Sensitivity	94	82.3	73.5
Specificity	75.9	59.3	55.6
Efficiency of prediction	82.9	68.2	62.5
Error of prediction	17.1	31.8	37.5
Probability of misclassification	18.2	25.7	28

الملخص العربى

تقييم انتيجين محضر بالساركوسيل من الميكوبكتيريم بوفيس لتشخيص السل البقرى فى الجاموس

* عصام أمين نصر * دانيال جندى ميخائيل

* عبد السلام زكى حسين ** حامد محمود همام

* معهد بحوث الأمصال واللقاحات البيطرية – العباسية

** المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية – العباسية

تم تجميع عدد ثمان وثمانون عينة سيرم من الجاموس منها خمسون من حالات إيجابية لاختبار التيوبركلين المفرد بالجلد وثمان وثلاثون من جاموس سالب لنفس الاختبار والمستخدم فيه نوعان من التيوبركلين النقى للماشية المستخرج من كل من العترة الآدمية والعترة البقرية لميكروب السل.

وقد أستخدم اختبار الاليزا المعدل غير المباشر وكانت الأنتيجينات المستخدمة هى ال ب.ب.د. بنوعيه الآدمى والبقرى وانتيجين مستخلص بواسطة الساركوسيل.

وأظهرت النتائج أن انتيجين الساركوسيل قد أعطى أفضل النتائج فى التمييز بين الحيوانات المصابة والحيوانات التى ليس بها إصابة سلية، وكانت قيم الحساسية والخصوصية وكفاءة التوقع أعلى من مثيلاتها فى حالة استخدام ال ب.ب.د بنوعيه. كذلك كانت احتمالية التصنيف الخطأ ١٨% من الحيوانات المختبرة باستخدام انتيجين الساركوسيل بينما كانت ٢٨% و ٢٥,٧ % مع ال ب.ب.د بنوعيه على التوالى.

وبهذا تتضح أهمية استخدام الأنتيجينات المحسنة فى اختبار الاليزا مثل انتيجين الساركوسيل كمكمل لنتائج اختبار التيوبركلين المفرد فى الجلد لزيادة كفاءة برامج مكافحة مرض السل البقرى فى الحيوانات.