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Mycobacterium bovis tuberculins: Preparation and antigenic properties

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Abstract: Three types of tuberculin were prepared from one isolate of *M. bovis* which was isolated from clinically tuberculosis cow. The isolate was purified and identified after it had been subjected to all biochemical tests and laboratory animal inoculation. These tuberculins include:
The purified protein derivative, new tuberculin and lysozymal extract.
The preparations were subjected to different *in Vivo* and *in Vitro* tests of potency and specificity .The results showed that the lysozymal extract had the highest potency in the diagnosis of the diseases in the experimentally infected animals, while the other tuberculin (purified protein derivative and new tuberculin) were equal in their potency as that of the imported one .

Key words: *mycobacterium bovis,* tuberculins, preparations, antigenic analysis.

تحضير السلينات من عصيات السل البقري مع دراسة بعض خواصها الاستضدادية

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الخلاصة: حضرت ثلاث أنواع من السلينات من عزلة محلية لجرثومة *M. bovis* من بقرة شخصت أصابتها بالسل سريريا واجري على هذه العزلة كافة الفحوصات الكيمياحيوية المطلوبة وحقن الحيوانات المختبرية. أستخدمت هذه العزلة لتحضير : سلين مشتقات البروتين النقي والسلين الجديد ومستخلص اللايسوزايم .أخضعت هذه السلينات لكافة فحوصات القدرة والتخصصية في الزجاج وداخل الجسم الحي أتضح من هذه الفحوصات أن مستخلص اللايسوزايم ذا قدرة عالية في تشخيص المرض في الحيوانات المصابة تجريبيا وقد تفوق على سلين مشتقات البروتين النقي والسلين الجديد ، بينما تقاربت قدرة سلين مشتقات البروتين النقي المحلي والسلين الجديد مع قدرة نظائرهما من السلينات المستوردة .

Introduction

Tuberculins have been used for the diagnosis of tuberculosis in cattle for more than 100 years. Purified protein derivative (PPD) tuberculins are most widely used today (1). Although PPD are described as pure, they are complex mixture of proteins, lipids, sugars and nucleic acids including different types of antigens which are common to several mycobactrial species (2).

The production of PPD involves three procedures that denature the antigens: autolysis, heating to 100^0C and precipitation of proteins, and this is why they are of low specificity (3, 4, 5). Several trials on the development and evaluation of antigens specific to *M.bovis* were used to improve the potency and specificity of the test (6,7,8,9).

The purpose of the present study was to:

A. Prepare three types of tuberculins including PPD, new tuberculin and lysozymal extract, of virulent local strain of *M.bovis*.

B. Compare the potency of each of the three tuberculins and imported bovine PPD for detecting delayed type hypersensitivity (DTH) responses in *M.bovis* and BCG sensitized guinea pigs and in naturally tuberculous cattle and evaluate the locally prepared tuberculins *invitro* by passive haemagglutination and haemagglutination inhibition tests.

Materials and Methods

1- Collection of materials:

Mediastinal lymph nodes with gross visible lesion were collected from reactor cattle which had been slaughtered in a dairy cattle station around Baghdad .sterile material and equipment was used for collection and handling of samples. Samples were transported to the laboratory in a cold container .Specimens were brought to the laboratory and impression smears were immediately prepared, air dried, fixed and stained with Ziehl – Neelsen stain and examined for acid – fast rods (10).

a- Processing of tissue:

A portion of tissue was processed as described by Claxton *et al* (10) and inoculated into stonbrink's media tube (at least two tubes). The tubes were incubated at 37^0C for 8-12 weeks when tubes showed evidence of growth, smears were made and examined for acid fast bacteria (11) .

b- Typing of *M.bovis*

For typing of mycobacteria, different biochemical tests were considered (12) and differential pathogenicity for guinea pigs and rabbits were performed (13).

2- *Preparation of tuberculins*:

a. Purified protein derivative, by the method described by Angus (14).

b. New tuberculin, by the method described by Stanford *et al* (6).

c. lysozymal extract , by the method described by Hall and Thoen (7).

d. protein concentration of crude tubceculins were estimated following the method described by Bradford (15).

3- Potency tests:

a. In sensitized guinea pigs

Tests for potency and standardization of locally prepared extract were performed as described by WHO (16).

To render guinea pigs tuberculin sensitive, several methods were adopted

i. By s/c injection with one human dose of freeze dried Japan BCG (Japan laboratories ltd. Japan).

ii. I/M injection of 2mg. heat killed *M.bovis*. Sensitization was accomplished in 4 weeks and skin testing was then started by injection of 0.1 ml of the locally prepared PPD, New tuberculin, lysozymal extract and a reference preparation of tuberculin bovine PPD (30.000 1.U/ml, 1 mg/ml. Lelystad /Holland).

Three dilutions (0.04, 0.2, 1 µg/ml) of those reagent were injected intra–dermal along the shaved sides of (20)

guinea pigs. A control group of non – sensitized guinea pigs was included and received similar doses of the each extracts and a standard PPD. Buffer control was included.

The injection sites on all guenia pigs were observed at 4, 24 and 48 hours after injection and mean diameter of the response were recorded. A standard graph was made by plotting the diameters of reaction against the logarithm of the concentration of the dose (17).

b. In naturally tuberculous cattle .

Field trial, the locally prepared extracts were performed on eight tuberculous cow (*M.bovis* was isolated form milk and nasal swabs). Each cow received an I/D injection of 0.1 ml (20 and 100µg /ml) of the locally prepared extracts and a reference bovine PPD on separate sites on the neck.

Results were interpretated by increase in the skin fold thickness 24, 48 and 72 hours after injection.

All results were subjected to statistical analysis using the f-test and the analysis of variance (18).

c.indirect haemagglutination test:

This was performed following the method described by Boyden (19) using serum of naturally tuberculous cow.

d.Haemagglutination inhibition reaction (HIR):

This was performed according to the method used by kwapinski (20).

Results and Discussion

Bacteriological Results

Both tests of pathogenicity and typing have confirmed characteristics of *M. bovis* of isolates form L. Nodes. All rabbits and guinea pigs died after 10 weeks after inoculation and an acid fast bacilli was isolated from all visceral organs.

Specificity and relative potency of prepared tuberculin's

Interest in *M.bovis* antigens, from the veterinary point of view, has been mainly directed towards developing better diagnostic test, (9, 21).

Guinea pigs sensitized with mycobacteria and naturally infected cattle with *M. bovis* have been used in comparing tuberculin (22, 23, 1) results showed that, at 24 hours, DTH responses in *M.bovis* and BCG sensitized guinea pigs were elicited by a locally PPD, new tuberculin, lysozymal extract and a reference PPD (figure 1and 2).

Lysozymal extract of *M.bovis* induced significant greater DTH responses than did locally PPD, new tuberculin and a reference PPD at each of the protein concentration of 0.04, 0.2 µg ($P<0.05$). There were no significant differences in skin test responses observed using a bovine or the locally PPD and new tuberculin at equal protein concen. ($P>0.05$).Significant differences in DTH responses were elicited by a locally PPD and by lysozymal extract at the protein conc. of 1µg ($P<0.05$), while no significant differences in DTH responses were observed between PPD of *M.bovis* and new tuberculin at the protein conc. of 1µg ($p>0.05$).

Skin test response were not observed in the non sensitized guinea pigs at 4, 24, and 48 hours. Buffer failed to induce DTH reaction in guinea pigs sensitized with *M.bovis* and BCG or in controls groups.

The locally prepared extracts were found to be safe and steril when inoculated into blood agar and stonbrink's medium and they caused no

illness when injected into the animals. The result of clinical testing of lysozymal extract, new tuberculin and locally PPD are summarized in (Table1).

Eight cows gave variable positive skin reaction to all these extracts and reference PPD. Responses were significantly greater at 48 and72 hours than responses at 24 hours at protein conc. 20 and 100µg (P>0.05), significant greater responses were observed by locally PPD and lysozymal extract at 48h at conc. 100µg (P<0.05). The skin test response to locally PPD and lysozymal extract were significantly greater than the response to arefrence PPD at a conc. 20µg at 72h (P<0.05) while at a cone 100µg the skin response to locally PPD was significant greater to arefrence PPD new tuberculin and lysozymal extract (P<0.01) at 72h in the second experiment the mean increases of skin fold was proportional to the logarithm of the doses injected (Table 1 and 2).

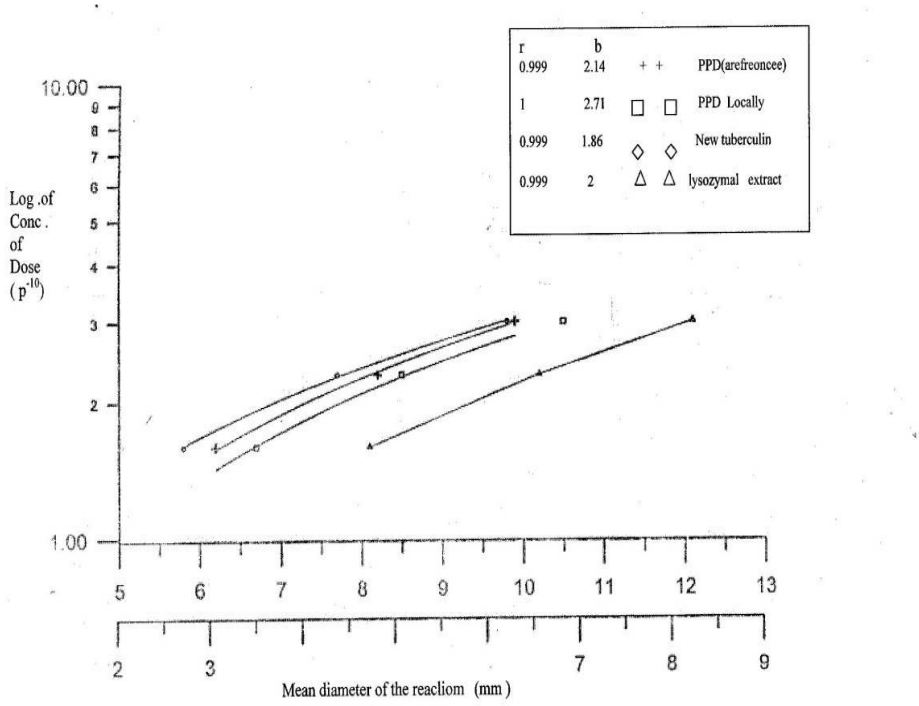

Figure (1) Relationship between the logarithm of the concentration of the dose and the mean diameter of the reaction (mm) for different prepared tubesculine in guinea pigs sensitized with BGG

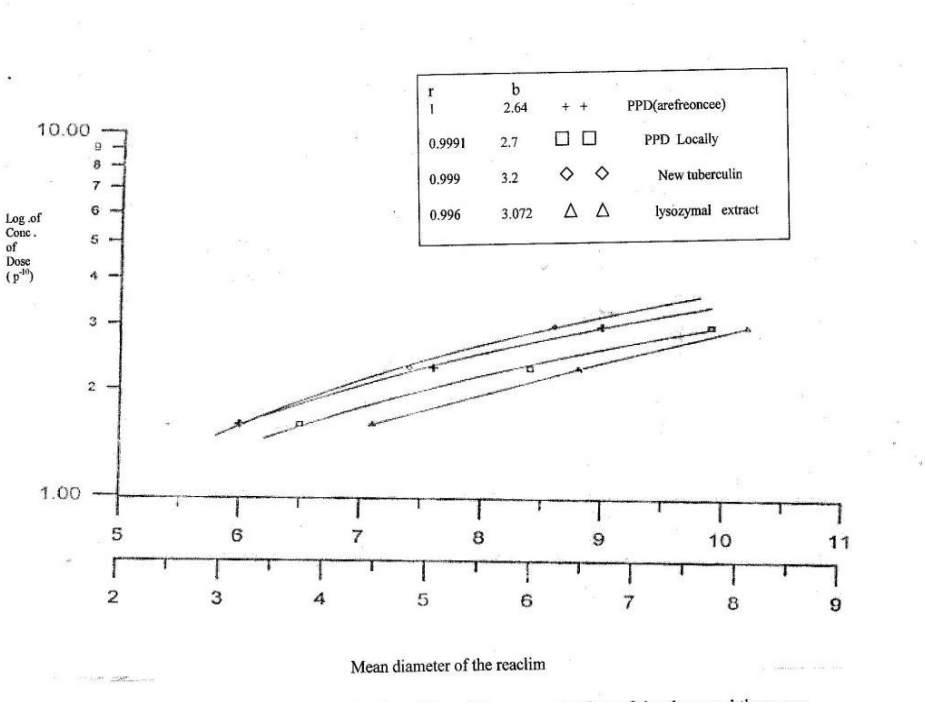

Figure (2): Relationship between the logarithm of the concentration of the dose and the mean diameter of the reaction (mm) for different prepared tuberculin in guinea pigs sensitized with BCG

Table 1: Mean increases of skin fold thickness of naturally tuberculous cattle injected with different dilutions (µg/ml) of different tuberculins

Type of tuberculins	**(M±SE) increases of skin fold thickness (mm)**					
	After 24 h		**After 48 h**		**After 72 h**	
	Dilution (µg/ml)		**Dilution (µg/ml)**		**Dilution (µg/ml)**	
	20	100	20	100	20	100
Arefrence purified protein derivative (PPD)	5.4±0.5	6±0.5	8.5±1.7	11.1±1.6	11.5±0.5	16±1
	a	a	a	a	a	a
A locally purified protein derivative (PPD)	5±0.5	5.8±0.9	8.9±2.5	12±0.8	13±1.1	18.3±1.1
	a	a	a	b	ab	b**
New tuberculin (NT)	5.3±0.4	5.8±0.6	9.3±2.1	11.3±1.5	11.4±0.9	14.1±2.9
	a	a	a	a	a	a
Lysozymal extract	5.2±0.3	5.6±0.2	11.6±1	12.6±1.2	14.1±2.6	16.2±2
	a	a	b	b	b	a

small letters means ($p<0.05$).
**means ($p<0.01$).

Table 2: Regrration & correlation coefficient between mean increases of skin fold of naturally tuberculous cow and log of the conc. of the dose of different tuberculins

Type of tuberculin	Regrration coefficient (b)		Correlation coefficient (r)
	Log. Of conc. (20)	Log. Of conc. (100)	
Arefrence purified protein derivative	0.85	1.15	0.99
Locally purified protein derivative	1.83	1.09	0.96
New tuberculin (NT)	1.75	2.07	0.95
Lysozymal extract	1.64	0.67	0.98

Acid fast rods was isolated from milk and nasal swabs from all of them which have characteristics of *M.bovis*. It has been recommended that the potencies ratio of PPD or other mycobacterial antigen preparation comparison with a reference standard vary with species tested and route of exposure (24). This may explain the differences in guinea pigs skin test responses observed and some variability in skin reactivity observed in naturally exposed cattle in skin responses using each of the prepared extracts.

In this study the potency of lysozymal treatment of delipidated cell walls of mycobacteria was significantly greater than that of an autoclaved / PPD of *M.bovis*, therefore autoclaving of PPD may have denatured or altered PPD components that induce DTH responses, However, additional loss of antigenicity was observed as measured by cross Immunoelectrophroesis after culture filtrates or sonified extracts and lysozymal extracts were autoclaved (7, 24).

Antigenic analysis of all locally prepared extracts and a reference PPD showed that, the locally PPD and arefrence PPD inhibit agglutination between RBC'S sensitized with M.bovis 0.5 mg/ml and *M.bovis* agglutinate (1:5000) at protein concentration 0.00024, 0.00014 mg /ml respectively. New tuberculin inhibit agglutination at protein conc. 0.0059 mg/ml. while lysozymal extract inhibit agglutination at protein concentration 0.017 mg /ml (figure.3).

Results suggest that components present in these preparation or in a reference PPD that induce DTH responses may differ from components present in humoral responses as detected by HI activity. (25)

Conclusion

In this study culture filtrates and sonic disrupted whole–cell and lysozymal extracts preparations have been used to produce soluble mycobacteria extracts from a local isolate of *M.bovis* which may help greatly in the diagnosis & eradication of the disease in the country, since the definitive characterization of the components present in these antigen preparation is limited and the antigenic activity of these crude antigen were compared with arefrence PPD *in vitro* as judged by the haemagglutination inhibition activity. This study was proved that those locally prepared tuberculins have specific antigenic and biological properties which could be used as a diagnostic tool in the diagnosis of the diseased and the methods adopted in the preparation of there tuberculin are simple, save and cheap.

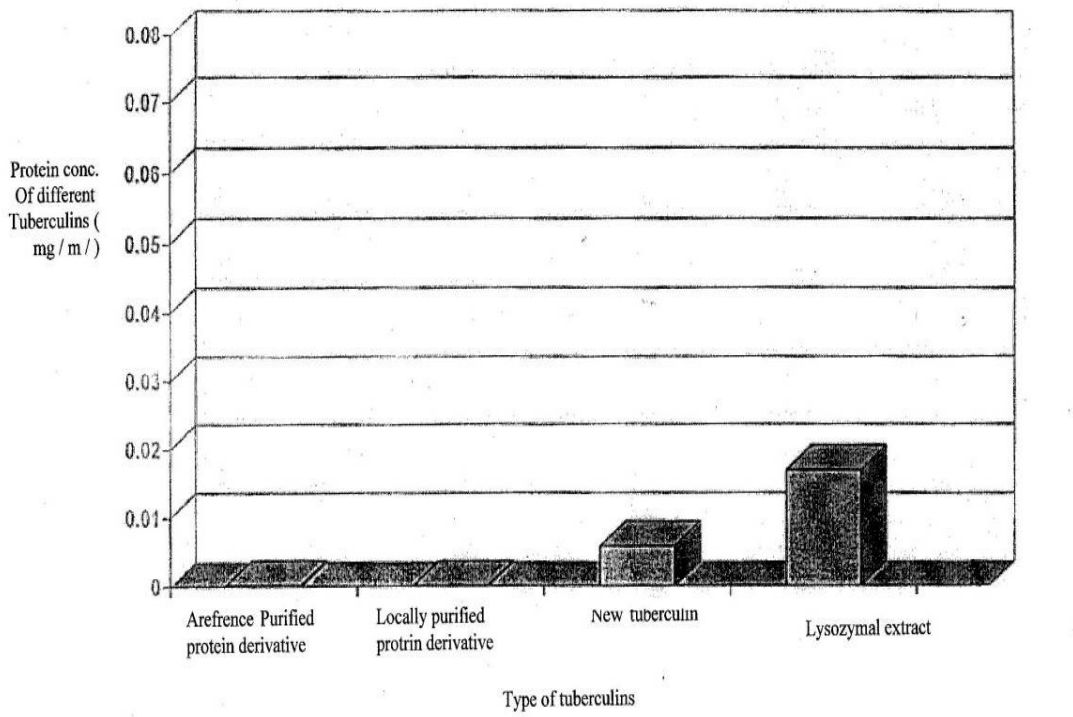

Figure (3): The required protein concentration of tuberculins to induce inhibition agglutination between sheep RBcs' sensitized with *M.bovis*

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