Assessment of a PPD tuberculin produced from the BCG strain of *Mycobacterium bovis* for use in cattle

A. NADER *, I.N. DE KANTOR **, V. RITACCO ***, J. AUGIER **** and F. ROMAIN ****

Summary: The possibility of using BCG — a *Mycobacterium bovis* strain of attenuated virulence — for PPD production instead of a virulent strain of *M. bovis* has been considered to avoid the biological risks involved in the procedure. The potency and specificity of a PPD tuberculin prepared from BCG was compared in guinea pigs with that of PPD produced from strain AN5 (two batches), a virulent strain of *M. bovis*: the European Communities Standard (ECS), and a reference batch prepared at the Pan American Zoonoses Center (CPZ). The potency of the BCG-PPD was also assessed in cows naturally infected with *M. bovis* in comparison with the other two PPD and a third (WS) produced at the Central Veterinary Laboratory, Weybridge, United Kingdom. Finally, the antigenic value of BCG-PPD was compared with that of CPZ and ECS in an ELISA against sera from tuberculous and healthy cattle. In the above assays, BCG-PPD showed a lower sensitivity and specificity than PPD produced from virulent *M. bovis*. Therefore its use either for tuberculin testing of cattle or as antigen in the ELISA test for detecting bovine IgG anti-*M. bovis* antibodies would not seem advisable.

KEYWORDS: Bovine tuberculosis - Cattle - *Mycobacterium bovis* - PPD tuberculin.

INTRODUCTION

Tuberculin testing of cattle is currently carried out with PPD (purified protein derivative) tuberculin produced from *M. bovis* strain AN5, which is virulent for human beings and bovines.

Several hundred litres of mycobacterial cultures may be processed annually by a laboratory to prepare bovine PPD. Although appropriate safety measures are observed, the handling of such a large amount of virulent tubercle bacilli always entails some risk.

---

** Pan American Zoonoses Center (CEPANZO/PAHO/WHO), C.C. 3092, 1000 Buenos Aires, Argentina.
*** University of Luján, C.C. 221, Luján, Argentina.
**** Tuberculins Unit, Pasteur Institute, 75724 Paris Cedex 15, France.
Argentina has a cattle population estimated at approximately 50 million and the average national prevalence of tuberculous animals has been estimated to be 4.3%. Increasing quantities of tuberculin doses are needed every year for screening and diagnostic tests in areas infected by tuberculosis (TB) and for the accreditation of TB-free herds.

A proposal made by the WHO Division of Biologicals to test a new batch of PPD tuberculin produced from *M. bovis* BCG (a strain of attenuated virulence) kept at the Pasteur Institute in Paris, and kindly provided by that institution, was investigated by the Argentinian authorities responsible for controlling bovine TB. A tuberculin prepared from BCG, if successful in picking out TB-infected cattle, would be of practical value for the production process. Therefore, the National Service of Animal Health, the University of Luján, and the Pan American Zoonoses Center (CEPANZO, PAHO/WHO) undertook a cooperative project to assess the potency and specificity of this new PPD batch.

**MATERIAL AND METHODS**

To test the potency and specificity of this PPD batch produced from BCG, it was used as an antigen in three different assays:

1. Intradermal tests in guinea pigs for potency and specificity determinations.
2. Intradermal tests in cattle for potency determination.
3. Enzyme immunoassay (ELISA) for detecting bovine IgG anti-*M. bovis* antibodies.

**PPD tuberculin**

The following freeze-dried bovine PPD tuberculins, reconstituted to 1 mg of protein per ml, were used:

- European Communities Standard PPD, prepared from strain AN5 at the Central Veterinary Laboratory of the Netherlands (ECS);
- Batch 291, Working Standard, prepared from strain AN5 at the Central Veterinary Laboratory, Weybridge, Surrey, UK (WS);
- Batch 86-84, produced from strain BCG at the Pasteur Institute, Paris (PI) and Batch 1-85, produced from strain AN5 at CEPANZO, Buenos Aires, supplied in liquid form, 1 mg/ml (CPZ).

To prepare the injectable solutions for assay purposes, all tuberculins were diluted with isotonic phosphate-buffered saline (PBS) at pH 7.3.

**Assay in guinea pigs**

a) *Relative potency*: The relative potency of PI in comparison with CPZ and ECS tuberculins was tested in twelve albino guinea pigs (500-700 g weight) four weeks after each animal had been inoculated with 0.001 mg *M. bovis* living strain AN5, suspended in PBS, by the intramuscular route. Three dilutions of each PPD were used: 5.0, 1.0 and 0.2 µg/ml.
b) Specificity: The same three PPD preparations as above and PPD *M. avium* 1-85, produced at CEPANZO, were inoculated into twelve guinea pigs having similar characteristics to those used in the potency test, but sensitised with killed *M. avium* strain D4 suspended in mineral oil (2). Two injection strengths of each PPD were used: 5.0 and 1.0 µg/ml. Tests were carried out as previously described (5, 6).

Assay in cattle

A first assay was performed in twenty-seven naturally-infected cows from a dairy herd at Navarro (Province of Buenos Aires).

All of these animals had shown a positive reaction to bovine PPD three months earlier. CPZ, WS and PI tuberculins were used at injection strengths of 1.0 and 0.2 mg/ml; ECS was used at 0.5 and 0.1 mg/ml.

In each case a 0.1 ml dose of each of the eight tuberculin dilutions was injected intradermally into each cow. The distance between the injection sites was 10-12 cm. Two dilutions of ECS and WS were inoculated on the left side of the neck of thirteen cows and two dilutions of each of the other two PPD on the right side. The remaining fourteen cows were also given two dilutions of each of the four tuberculins, but the inoculation sites were allocated in reverse (ECS and WS on the right side, PI and CPZ on the left side).

The thickness of the skin fold at each injection site was measured with calipers and recorded, as previously described (5), before the injection and 72 hours afterwards.

A second assay was conducted to confirm the potency relationship between CPZ and PI obtained in the previous assay. Sixteen naturally-infected cows from the same dairy herd were inoculated with 0.1 mg of CPZ and 0.4 mg of PI, both in a 0.1 ml volume, on the right side of the neck 10-12 cm apart. Each PPD was injected at an upper site in eight cows and a lower site in the other eight.

Reactions were read as previously described and the mean reactions to each tuberculin were compared.

Enzyme-linked immunosorbent assay (ELISA)

Tuberculins CPZ, ECS and PI were used as antigens in an ELISA designed for detecting bovine IgG antibodies to *M. bovis*. Serum samples were collected from:

(a) twenty cattle with tuberculous lesions at necropsy confirmed by isolation of *M. bovis*; and (b) twenty-two tuberculin-negative cattle from a tuberculosis-free area.

The ELISA procedure has been described elsewhere (4). Briefly, microtitre plates sensitised with each antigen at 0.01 mg/ml in carbonate buffer at pH 9.6 were incubated in duplicate with test sera diluted 1:200, followed by incubation with rabbit antibovine IgG conjugated with peroxidase (Accurate Chem. and Sci. Corp., Westbury, NY, USA). The enzymic substrate chromogen (H$_2$O$_2$-ABTS) was added after washing the wells, and incubated at room temperature for 20 minutes. The reaction was stopped with hydrofluoric acid. Optical densities (OD) were assessed at 405 nm on a micro-ELISA reader.
Statistical analysis

Results of intradermal tests were analysed by parallel line assay (3). Mean tuberculin tests in the second assay in cattle were compared by the test. Correlation coefficients were calculated for the analysis of the ELISA results.

RESULTS

Assay in guinea pigs

a) Relative potency: Potency values expressed as a percentage, with 95% confidence limits, were 64.6 (39.8-102.0) for CPZ versus ECS, and 8.3 (4.1-14.5) for PI versus ECS.

b) Specificity: Specificity expressed in terms of percentage of potency of the tested bovine PPD against the avian PPD in guinea pigs sensitised with M. avium, with 95% confidence limits, were: 7.4 (2.6-11.4) for ECS, 2.8 (0.7-6.3) for CPZ and 18.2 (8.3-30.2) for PI.

Assay in cattle

The results of the first assay are presented in Table I. The potencies of CPZ, WS and PI are given relative to that of ECS. Detailed information on test results is given in Table II.

TABLE I

Assays in 27 naturally-infected cattle: potency (%) relative to the European Communities Standard bovine PPD (ECS) (95% confidence limits)

<table>
<thead>
<tr>
<th>PPD tuberculins</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEPANZO 1-85 (CPZ)</td>
<td>97.7 (61.7-151.0)</td>
</tr>
<tr>
<td>Weybridge 291 (WS)</td>
<td>38.9 (11.5-61.7)</td>
</tr>
<tr>
<td>Pasteur Institute 86-84 (PI)</td>
<td>13.8 (1.3-28.8)</td>
</tr>
</tbody>
</table>

In the second assay mean reactions for CPZ (0.1 mg) and PI (0.4 mg) were respectively 6.7 and 6.4 (standard deviations 4.4 and 5.2). The difference was not significant.

Enzyme-linked immunosorbent assay

Except for a few sera with high antibody levels, the optical density (OD) obtained with CPZ showed a very close parallelism to that of ECS; the former were slightly higher (r: 0.97; Figure 1).

On the other hand, correlation between values obtained with PI and ECS was lower (r: 0.76; Figure 2).
**TABLE II**

Assay in 27 naturally-infected cattle. Mean skin fold thickness increases (Y), standard deviations (SD), Sy, Sy² values and regression equations (RE)

<table>
<thead>
<tr>
<th></th>
<th>ECS Standard bovine PPD</th>
<th>Weybridge batch</th>
<th>CEPANZO batch 1-85</th>
<th>Pasteur Institute (BCG) batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{X} ) (mg/ml)</td>
<td>0.5</td>
<td>0.1</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>( \bar{Y} ) (mm)</td>
<td>6.8</td>
<td>4.9</td>
<td>6.4</td>
<td>4.8</td>
</tr>
<tr>
<td>SD</td>
<td>3.7</td>
<td>3.3</td>
<td>2.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Sy</td>
<td>183.5</td>
<td>132.1</td>
<td>173.2</td>
<td>128.8</td>
</tr>
<tr>
<td>Sy²</td>
<td>1599.5</td>
<td>925.5</td>
<td>911.4</td>
<td>800.5</td>
</tr>
<tr>
<td>RE</td>
<td>5.14 + 2.35x</td>
<td>4.18 + 2.35x</td>
<td>5.12 + 2.35x</td>
<td>3.11 + 2.35x</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study, the biological potency and the specificity of a PPD prepared from the attenuated strain BCG (PI tuberculin) were lower in guinea pigs than the potency and specificity of two PPD tuberculins produced from a virulent strain of *M. bovis* (the European Communities Standard (ECS) and the CEPANZO Reference preparation (CPZ)).

When tested in naturally-infected cattle, the PPD produced from BCG was less potent than ECS, CPZ and the Weybridge Working Standard bovine PPD (WS).

When assessed as antigens in a serological test (ELISA) against sera from tuberculous and healthy cattle, a high correlation was observed between the results obtained with CPZ and ECS. On the other hand, when PI was used as antigen, results correlated poorly with those of ECS. This indicates that the sensitivity and specificity of ELISA would be lower if PI were used as antigen.

The poorer sensitivity and specificity of a PPD prepared from BCG as compared with those produced from a typical *M. bovis* strain could be related to an antigenic modification of BCG, which might have occurred when the originally virulent *M. bovis* mutated to an attenuated form (2). In fact, a high degree of cross-reactivity between antigens from BCG and those of mycobacterial species other than *M. bovis* has been demonstrated (1).

Even though cellular and humoral immune mechanisms do not necessarily follow identical paths, clear parallelism was observed in this study between cutaneous hypersensitivity and the humoral recognition of the investigated antigens.
Fig. 1

Anti-M. bovis antibody levels in 42 bovines as determined by ELISA (405 nm). Correlation between results obtained with PPD CPZ (CEPANZO) and PPD ECS (European Communities Standard).
Each point represents one animal
+ healthy cattle
* tuberculous cattle

**FIG. 2**

Anti-*M. bovis* antibody levels in 42 bovines as determined by ELISA (405 nm). Correlation between results obtained with PPD BCG (Pasteur Institute) and PPD ECS (European Communities Standard)

Resumen: A fin de evitar el riesgo biológico asociado a la producción de tuberculina PPD, ceci afin d'éviter les risques biologiques inhérents à la méthode. La puissance et la spécificité de la tuberculine PPD, preparada con el BCG (PPD-BCG), son été comparadas en cobayas, a celas de dos antoquinas PPD, produites a partir de la souche AN5, souche virulente de M. bovis : la tuberculine standard des Communautés Européennes (CE), et un lot de référence du Centre panaméricain des zoonoses (CPZ). L'évaluation de la puissance de la tuberculine PPD-BCG, effectuée sur des vaches infectées naturellement, s'est faite par comparaison avec les deux tuberculines PPD indiquées ci-dessus, ainsi qu'une troisième (WS), produite au Laboratoire Vétérinaire Central de Weybridge (Royaume-Uni). Enfin, la valeur antigénique de la tuberculine PPD-BCG a été comparée à celle du CPZ et des CE, par le test ELISA, vis-à-vis de sérum de bovins tuberculeux et d'animaux sains. La tuberculine PPD-BCG s'est avérée, dans les essais ci-dessus, d'une sensibilité et d'une spécificité moindres que la tuberculine PPD produite à partir d'une souche virulente de M. bovis. C'est pourquoi son emploi pour la tuberculization de los bovins, ou comme antigène dans le test ELISA para la detección de anticuerpos IgG anti-M. bovis, ne semble pas à conseiller.

PALABRAS CLAVE: Bovinos - Mycobacterium bovis - Tuberculina PPD - Tuberculosis bovina.

**

**

ÉVALUATION D'UNE TUBERCULINE PPD A USAGE BOVIN PRÉPARÉE A PARTIR DE BCG. — A. Nader, I.N. de Kantor, V. Ritacco, J. Augier et F. Romain.

Résumé : Les auteurs ont étudié la possibilité d'utiliser le BCG — souche attenuée de Mycobacterium bovis —, pour la production de tuberculine PPD, ceci afin d'éviter les risques biologiques inhérents à la méthode. La puissance et la spécificité de la tuberculine PPD, préparée avec le BCG (PPD-BCG), ont été comparées sur cobayes, à celas de deux autres tuberculines PPD, produites à partir de la souche AN5, souche virulente de M. bovis : la tuberculine standard des Communautés Européennes (CE), et un lot de référence du Centre panaméricain des zoonoses (CPZ). L'évaluation de la puissance de la tuberculine PPD-BCG, effectuée sur des vaches infectées naturellement, s'est faite par comparaison avec les deux tuberculines PPD indiquées ci-dessus, ainsi qu'une troisième (WS), produite au Laboratoire Vétérinaire Central de Weybridge (Royaume-Uni). Enfin, la valeur antigénique de la tuberculine PPD-BCG a été comparée à celle du CPZ et des CE, par le test ELISA, vis-à-vis de sérums de bovins tuberculeux et d'animaux sains. La tuberculine PPD-BCG s'est avérée, dans les essais ci-dessus, d'une sensibilité et d'une spécificité moindres que la tuberculine PPD produite à partir d'une souche virulente de M. bovis. C'est pourquoi son emploi pour la tuberculization des bovins, ou comme antigène dans le test ELISA para la detección de anticuerpos IgG anti-M. bovis, ne semble pas à conseiller.

MOTS-CLÉS : Bovins - Mycobacterium bovis - Tuberculine PPD - Tuberculose bovine.

**

**


Resumen: A fin de evitar el riesgo biológico asociado a la producción en gran escala de tuberculina PPD a partir de una cepa virulenta de M. bovis, tal como la cepa AN5, corrientemente empleada, se consideró la posibilidad de emplear en su lugar M. bovis BCG, de virulencia atenuada. En el presente estudio se compararon en cobayas la sensibilidad y especificidad de un PPD preparado con BCG (BCG-PPD) con dos PPD producidos con la cepa AN5: el PPD estándar de la Comunidad Económica Europea (ECS) y un lote de referencia preparado en CEPANZ (CPZ). La potencia del BCG-PPD también se evaluó en vacas con infección natural por M. bovis, en relación con los dos PPD antes mencionados y un tercero producido en el Laboratorio Veterinario Central de Weybridge, Inglaterra (WS). Finalmente, el BCG-PPD se comparó con CPZ y ECS usados como antígenos en ELISA, frente a sueros de ganado bovino tuberculoso y no tuberculoso. En estas experiencias el BCG-PPD mostró sensibilidad y especificidad menores que los PPD producidos de una cepa virulenta de M. bovis. Por lo tanto no sería conveniente su adopción para pruebas tuberculínicas en ganado bovino o como antígeno en ELISA para detección de anticuerpos IgG bovinos anti-M. bovis.

PALABRAS CLAVE: Bovinos - Mycobacterium bovis - Tuberculina PPD - Tuberculosis bovina.

**

**
REFERENCES


