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A SIMPLE METHOD FOR THE EXTRACTION OF POLYSACCHARIDE B FROM BRUCELLA CELLS FOR USE IN THE RADIAL IMMUNODIFFUSION TEST DIAGNOSIS OF BOVINE BRUCELLOSIS

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Résumé

UNE MÉTHODE SIMPLE D'EXTRACTION DU POLYSACCHARIDE B DE CELLULES DE BRUCELLA UTILISABLE POUR LA DÉTECTION DES BOVINS INFECTÉS PAR LA TECHNIQUE D'IMMUNODIFFUSION RADIALE. — Pour extraire le polysaccharide B de brucella utilisé pour la détection des bovins infectés par la technique d'immunodiffusion radiale (RID), une méthode de préparation rapide, simple et sans danger est proposée. Elle utilise des cellules tuées par le phénol de la souche de *B. melitensis* 16 M stable pour la production de polysaccharide B. Après autoclavage des cellules puis centrifugation, le polysaccharide B est obtenu par précipitation en ajoutant au surnageant en deux étapes de l'éthanol. La comparaison des résultats des tests RID exécutés avec le polysaccharide extrait de *B. melitensis* 16 M (méthode rapide) et 231 sérums de bovins infectés montre la validité de cette méthode de préparation. Les différences de rendement (1,5 % pour le second précipité contre 0.5 % pour le polysaccharide-référence) et de dose optimale utilisée dans la réaction (10 µg contre 200 µg) montrent qu'on réalise 60 fois plus de tests RID avec le polysaccharide obtenu par la méthode rapide.

It has been shown recently (Jones *et al.*, 1980) that the use of a brucella polysaccharide antigen has made possible the identification of infected cattle in recently vaccinated herds which had high numbers of reactors to standard diagnostic test. A simple rapid radial immunodiffusion test developed by Diaz *et al.* (1979), and confirmed by Jones *et al.* (1980), has the potentiality of being more specific, and more economical to perform than other tests presently employed. The polysaccharide was obtained from rough *B. melitensis* 115 by trichloroacetic acid extraction and was designated polysaccharide B (Diaz and Levieux, 1972).

In the course of these studies two problems emerged.

1. The *B. melitensis* 115 strain was found to lose its ability to synthesize the polysaccharide B after repeated subculture and it was necessary to go back to the original dried strain.
2. The yield of polysaccharide B was low and the method somewhat cumbersome.

We knew the polysaccharide B was present in significant amounts along with smooth lipopolysaccharide (antigen A + M) in phenol-water extracts of smooth *B. melitensis* 16 M (Diaz *et al.*, 1968). In the present communication we describe a simple method for the pre-

paration of the polysaccharide B from *B. melitensis* 16 M. The identity of the antigenic determinant of this preparation with the polysaccharide B obtained from *B. melitensis* 115 cells employed previously, is demonstrated by immunodiffusion test and its effectiveness in RID test with sera obtained from infected bovines is comparable to the original polysaccharide B.

Materials and Methods

Antigenic preparations

B. melitensis 16 M cells were grown in Trypticase Soy Broth (BBL) flasks at 37 °C on a rotatory shaker. After 48 h of incubation the cells were killed by addition of 90 % phenol up to a final concentration of 0.5 % and incubation at 37 °C for 24 h. Repeated experiments showed that no viable cells are found in the flasks after this treatment. The phenol killed cells were harvested by centrifugation at 5 °C using a Lourdes « Betafuge » model A Centrifuge, equipped with a 3 RA rotor, at 12 000 × g for 30 min, washed twice with saline and resuspended in distilled water (30 g of packed cells in 100 ml of water). The resuspended cells were autoclaved at 120 °C for 30 min, cooled and centrifuged at 12 000 g for 30 min at 5 °C. The supernatant fluid was removed, mixed with three volumes of cold ethanol and held at 5 °C for 18 h with continuous stirring. The precipitate formed (first precipitate) was pelleted by centrifugation (5 000 g for 15 min at 5 °C) dissolved in distilled water, dialyzed

and freeze-dried. To the supernatant of this first precipitation two volumes of cold ethanol were added and the mixture kept at -20° C overnight. The resulting precipitate (second precipitate) was collected by centrifugation (5 000 g for 15 min), dissolved in water, dialyzed and freeze-dried. The yield was about 5 % for the first precipitate and 1.5 % for the second precipitate referred to the weight of the dry cells. Polysaccharide B from *B. melitensis* 115 was prepared by trichloroacetic acid extraction as described previously (Diaz *et al.*, 1979) (The yield of this method was about 0.5 %). Fraction 5 of Redfearn was prepared from *B. melitensis* 16 M by the modified phenol-water extraction method (Redfearn, 1960). The Fraction 5 contains both the lipopolysaccharide (antigen A + M) and the polysaccharide B (Diaz *et al.*, 1968).

Serological methods

Radial immunodiffusion and double immunodiffusion in agarose gels were performed as described previously (Diaz *et al.*, 1979). Tube seroagglutination (SAT) was performed as recommended by Alton and Jones (1967). Complement fixation (CF) titers of the sera used were kindly provided by Dr. E. Zarzuelo (Centro Regional de Brucelosis, Zaragoza, Spain) to whom sera are routinely sent for the official control of bovine Brucellosis.

Source of the sera

Bovine sera were obtained from cattle of the province of Navarra, Spain. For the present study we selected 231 sera from our collection

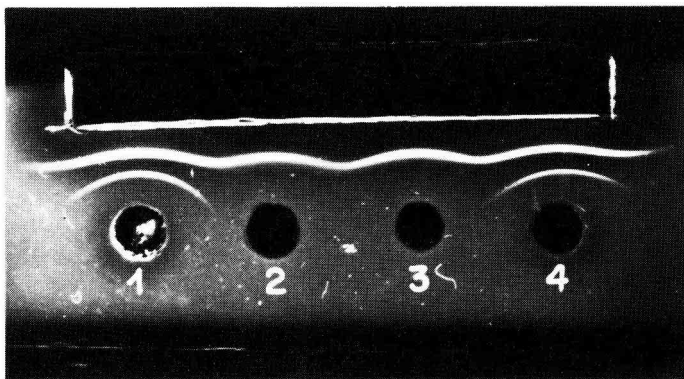


Fig. 1. — Gel diffusion of fraction 5 from *B. melitensis* 16 M (wells 1 and 4), polysaccharide B from *B. melitensis* (well 2) and second precipitate from *B. melitensis* 16 M reacted with sera from cattle infected with *B. abortus*.

Table 1. — Results of precipitation, SAT, CF tests and milk culture from ten cows giving different results in IDR and double gel diffusion.

Serum N°	IDR with poly B from <i>B. melitensis</i>		Double immunodiffusion with fraction 5 from <i>B. melitensis</i> 16 M		Tube agglutination titres (U.I.)	FC titre	Milk cultures
	115	16 M	Poly B	A + M			
1	+	+	+	—	16	160	<i>B. abortus</i>
2	+	+	+	—	16	160	Negative
3	—	+	+	+	508	160	<i>B. abortus</i>
4	—	+	+	+	128	160	Not done
5	+	+	—	+	64	160	<i>B. abortus</i>
6	+	+	—	+	64	40	Not done
7	+	+	—	+	25	40	<i>B. abortus</i>
8	+	+	—	+	64	80	<i>B. abortus</i>
9	+	+	—	+	32	40	Not done
10	+	+	—	+	32	40	Not done

previously shown to have antibodies to the polysaccharide B, by IDR using polysaccharide B from *B. melitensis* 115 (229 sera) or double immunodiffusion using the fraction 5 of Redfearn (2 sera).

Milk culture

Milk samples, when available, were inoculated on the selective medium described by Renoux, following the procedures described previously by Alton and Jones (1967).

Results

The results of the double immunodiffusion analysis of the first precipitate, showed two components, one with total identity to the lipopolysaccharide (antigen A + M) of fraction 5 and the other with total identity to both the polysaccharide B present in the fraction 5 and the *B. melitensis* 115 polysaccharide B. In contrast with the first precipitate the second precipitate showed only one component. This component displayed total identity with either the polysaccharide B of fraction 5 and the *B. melitensis* 115 polysaccharide B (Fig. 1).

The second precipitate did not show other component by double immunodiffusion even at a concentration as high 20 mg/ml, although a concentration as low as 9 µg/ml was enough to develop the polysaccharide B line. In contrast, *B. melitensis* 115 polysaccharide B had to be used at concentrations of at least 320 µg/ml in the double immunodiffusion test. Similar results were obtained when the RID

was used with individual sera placed in the wells. The optimal concentrations at which immunoprecipitate rings appeared within 3 h was 10 µg/ml of agarose for the second precipitate and 200 µg/ml of agarose for the *B. melitensis* 115 polysaccharide B. For the second precipitate some sera gave positive results at a concentration as low as 2.5 µg/ml, but never at concentrations higher than 200 µg/ml.

Further comparisons among the antigens were performed with either the RID or the double immunodiffusion technique using 231 sera known to be positive to polysaccharide B by either the RID test with *B. melitensis* 115 polysaccharide B or Ouchterlony with fraction 5 from *B. melitensis* 16 M. The optimal concentrations of 10 µg/ml of the second precipitate and 200 µg/ml of the *B. melitensis* 115 polysaccharide B were used in the IDR. The fraction 5 preparation was used at 5 µg/ml in the double immunodiffusion test.

All but 10 sera gave identical results, i.e. all were positive for the *B. melitensis* 115 polysaccharide B, the second precipitate and both components of the fraction 5, the lipopolysaccharide (antigen A + M) and polysaccharide B. Table 1 shows the results obtained with the 10 sera previously cited. All the 10 sera gave the characteristic precipitation line with the second precipitate, eight with the *B. melitensis* 115 polysaccharide B and four with the polysaccharide B present in fraction 5. Noteworthy, sera n° 1 and n° 2 did not show antibodies to the lipopolysaccharide (antigen A + M) while they showed antibodies to the poly-

saccharide B. For those two sera the CF titers were indicative of an active infection and the milk culture was positive in an animal, but the SAT titer were of 16 UI suggesting no infection.

Discussion

On the basis of the evidence presented we conclude that the second precipitate seem to be immunologically indistinguishable with the *B. melitensis* 115 polysaccharide B and with the polysaccharide B present in the fraction 5 of Redfearn. We are not able to show any other component in the second precipitate by double immunodiffusion, but the possibility of the presence of other components has not been rigorously ruled out. Nevertheless, our results show that this preparation is, for diagnostic purposes, at least as good as the *B. melitensis* polysaccharide B used in previous work (Diaz *et al.*, 1976; Diaz *et al.*, 1979; Jones *et al.*, 1980). The method described in the present communication for the preparation of polysaccharide B offers several advantages over the trichloroacetic acid extraction of *B. melitensis* 115 cells. First, *B. melitensis* 16 M is a reference strain easily kept in a stable form. Second, the method of extraction and differential precipitation is easy to perform, and it could be carried out in one laboratory equipped to handle pathogens. Since the *B. melitensis* 16 M is a pathogenic strain, precau-

tion have to be taken during inoculation and growth of the cells, although the killing of the cells with phenol allows handling them subsequently without any risk. Third, for the optimal doses used in RID with the *B. melitensis* 16 M polysaccharide B preparation it is possible to study twenty times more animals than with the preparation of polysaccharide B from *B. melitensis* 115. In our laboratory with the polysaccharide B obtained, from 35 g of *B. melitensis* 16 M dry cells, we are able to study theoretically 52 500 sera in contrast to the 875 with the polysaccharide B obtained from *B. melitensis* 115 R.

A rather remarkable result was the finding of two sera that did not show antibodies to the lipopolysaccharide (antigen A + M) whereas they had antibodies to the polysaccharide B. These two sera had SAT titers of 16 IU but CF titers of 1/160. It is known that there is a correlation between the presence of antibodies to the lipopolysaccharide and SAT titers in both human and cow sera (Diaz *et al.*, 1976; Panizo *et al.*, 1978; McMahon *et al.*, 1979). On the other hand Jones *et al.* (1980) have demonstrated that there is a correlation between CF titers and the ability of the sera to develop the polysaccharide B precipitation line. Altogether, these observations suggest that the polysaccharide B could play an important role in the CF test, and work must be done in order to confirm this hypothesis.

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Summary

In order to extract the polysaccharide B of brucella used in Radial Immunodiffusion test (RID) for detection of infected cattle, a quick, simple and safe method is proposed. It consists of using phenol killed cells of *B. melitensis* 16 M known to produce polysaccharide B. After autoclaving brucella cells resuspended in saline, the cells are separated by centrifugation and the polysaccharide B is obtained by two successive ethanol precipitations of the supernatant. Comparison of the results of RID tests carried out with the polysaccharide extract of *B. melitensis* 16 M (rapid method) and 231 sera of infected cattle shows the validity of this method of preparation. Differences in yield (1.5 % for the second precipitate against 0.5 % for the polysaccharide-reference and in optimum dose used the reaction (10 μ g against 200 μ g) show that as many as 60 times more RID tests can be made with the polysaccharide obtained by rapid method.

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