*Bull. Org. mond. Santé*
*Bull. Wld Hlth Org.* } 1958, **19**, 187-196

# THE USE OF GEL DIFFUSION PRECIPITIN PLATES IN THE STUDY OF BRUCELLA

W. BRUCE [1]
LOIS M. JONES [2]

*Central Veterinary Laboratory, Weybridge, Surrey, England*

SYNOPSIS

The use of gel diffusion precipitin plates in the study of brucella is described.

Cultures of *Brucella melitensis* yielded a diffusible precipitin antigen which produced lines with sera of rabbits, goats, and cattle that had been infected with either *Br. abortus* or *Br. melitensis*. The precipitin lines varied in number from one to three.

A diffusible precipitin antigen could not be prepared from *Br. abortus* and *Br. suis* cultures by the methods which were effective in producing such an antigen from *Br. melitensis*.

No precipitin reactions occurred with sera from cattle in brucella-free herds which had been inoculated with Strain 19 vaccine, although some of these sera were positive to the agglutination test.

The gel diffusion precipitin method originally developed by Ouchterlony in 1949 (Thesis, Karolinska Medico-Kirurgiska Institutet, Stockholm) and described by Bjorklund (1952) has been modified by Mansi (1957) for the study of virus antigen-antibody reactions.

This paper reports the application of the technique to the study of brucella. The precipitin plates were of the pattern described by Mansi, in whose paper a full description of their preparation will be found. Briefly, the technique consisted of pouring agar plates of a fixed depth into Petri dishes. When cool, four groups of holes were cut into the agar, each group consisting of a hexagon of six holes surrounding and equidistant from a centre hole. The diameter of the holes was 0.7 cm and the distance between them was 0.4 cm. After cutting, the bottom of each hole was sealed with a drop of molten agar to form a basin. The antigen was generally placed in the central basin of each group and the sera in those of the periphery. The plates were held at room temperature and readings were taken at 24 and 48 hours with the aid of a white light from a microscope lamp reflected by a concave mirror.

[1] Now Veterinary Research Officer, Department of Veterinary Services, Naivasha, Kenya, East Africa
[2] Now Consultant, Veterinary Public Health Section, World Health Organization, Geneva, Switzerland

## Study of Antigens

From smooth cultures of *Brucella melitensis*, including stock, avirulent and freshly isolated virulent strains, an antigen was readily obtainable which would diffuse and react in the precipitin plate when set up against positive brucella sera (see Fig. 1a). The following preparations of *Br. melitensis* were found to be antigenic:

1. The freshly harvested growth of two-day-old cultures on agar slopes.
2. The supernatant fluid from centrifuged cell suspensions in saline.
3. The extract from cells treated with 2.5% phenol for 24 hours.
4. The extract from cells treated with 12.5% trichloracetic acid for three hours in the cold.

The following treatments of the cell suspensions did not interfere with their antigenicity:

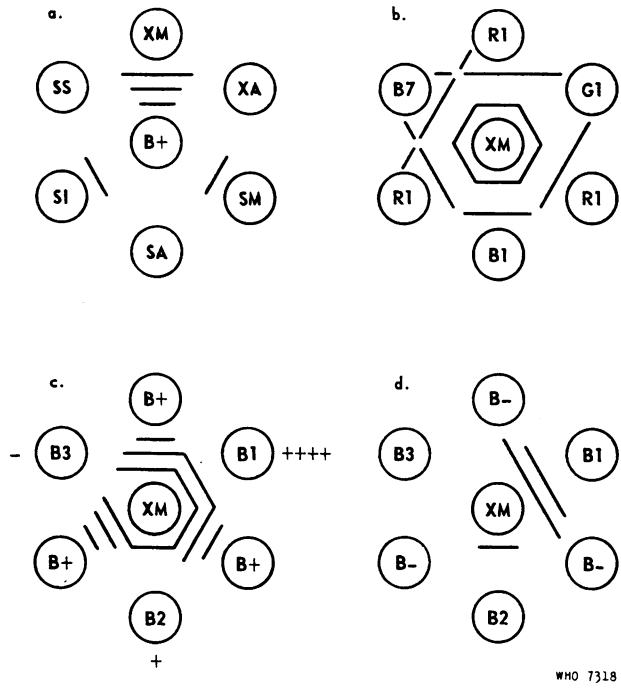
1. Heating at 60°C for one hour.
2. Boiling for 10 minutes.
3. Repeated rapid freezing and thawing between an alcohol, solid $CO_2$ bath at approximately —76°C and a water-bath at 37°C.
4. Treatment with ether for 48 hours at room temperature. The non-ether-soluble supernatant remained antigenic.
5. Treatment with N/20 NaOH for 72 hours at room temperature.

Similar preparations of six *Br. abortus* and two *Br. suis* cultures did not produce precipitin plate reactions with positive sera (Fig. 1a). However, the supernatant fluid from a heavy suspension of *Br. abortus*, which had been stored in 0.5% phenol saline in the cold for over six months, produced a line with one of three sera tested.

An atypical brucella culture which resembled *Br. abortus* in biochemical tests and *Br. melitensis* in agglutination tests with mono-specific sera produced a precipitin line (Fig. 1a).

The strength of the precipitin lines obtained with the *Br. melitensis* antigen varied with the preparation. Freshly harvested suspensions produced a diffuse line near the antigen basin. Cell suspensions after prolonged storage produced a stronger line. Any tendency towards rough colony production in a *Br. melitensis* culture reduced the precipitin antigen available in preparations. A culture of *Br. melitensis* which was antigenic when smooth no longer produced precipitin lines when it became 90% rough in broth culture. The trichloracetic acid extract of smooth *Br. melitensis* could be diluted as much as 1/32. Undiluted, or diluted 1/4, it formed three distinct lines with potent antisera and was used as the standard antigen throughout the study.

**FIG. 1. PRECIPITIN LINES OBTAINED WITH BRUCELLA ANTIGENS AND ANTISERA**




Antigens: XM = Trichloracetic acid extract of *Br. melitensis*
XA = Trichloracetic acid extract of *Br. abortus*
SM = Saline suspension of *Br. melitensis*
SA = Saline suspension of *Br. abortus*
SI = Saline suspension of *Br. abortus/melitensis*
SS = Saline suspension of *Br. suis*

Antisera: B+ = Positive Bovine Control Serum
B— = Negative Bovine Control Serum
R = Rabbit Serum
G = Goat Serum

## Study of Antibodies

Experimental or natural infection of animals with *Br. abortus*, *suis* or *melitensis* produced antibodies in the sera which gave positive reactions in the plate precipitin test when set up against the *Br. melitensis* antigen. Precipitins were stable at 60°C for two hours but were destroyed by boiling. Although some sera with agglutination titres greater than 1/320 (i.e., approximately 640 International Units) [1] could be diluted 1/40 without

[1] With the Weybridge agglutination methods used in this work, titres of 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320 equal approximately 20, 40, 80, 160, 320 and 640 International Units respectively (WHO Expert Committee on Biological Standardization, 1954; Stableforth, 1954).

losing precipitin activity, the strength of the line always decreased with dilution. Test sera were used undiluted and unheated.

In the study of virus diseases, Mansi (1957) was able to demonstrate the presence of antigens and antibodies simultaneously in the tissues of affected animals by placing tissue in the peripheral basins alternately with positive serum and with a standard antigen in the central basin. This method was followed in a limited trial with 21 minced tissues from each of three goats infected with *Br. melitensis*. Antibodies were demonstrated in the uterus of one goat and in the suprascapular lymph-node of another. Antigen lines were not produced with any of the tissues, although some were heavily infected with *Br. melitensis*. Antibodies were not demonstrable in the stomach, liver, lung, kidney, lymph-nodes, spleen or pancreatic tissues of several guinea-pigs infected with *Br. abortus*, but were demonstrable in their sera. A number of milk and whey samples which gave positive ring and agglutination tests failed to produce precipitin lines.

The plate precipitin test was compared with the agglutination and the complement-fixation test in the analysis of animal sera with various histories of infection. Standard negative and positive sera were set up with test sera in the pattern illustrated in Fig. 1c and 1d. A large supply of serum was drawn from a normal cow and from a cow infected with *Br. abortus* for the negative and the positive serum, respectively. This was frozen in small aliquots, stored at —20°C and thawed as required. The central basins were filled with the undiluted trichloracetic acid extract of *Br. melitensis*, which had been stored at 4°C. Alternate peripheral basins in one group (see Fig. 1c) were filled with undiluted bovine serum (B+), which produced three distinct precipitin lines. Alternate basins in the second group (see Fig. 1d) were filled with undiluted negative bovine serum (B—), which failed to produce lines. The test serum (B1, B2 or B3) was placed in one of the three remaining basins in each group. If a test serum produced a precipitin line, the intensity of the line could be compared with that of the positive serum and rated accordingly at the 48-hour reading: ++++ indicated that the line was the same length as that of the positive control, i.e., the sides of the hexagon were equal and there was a well-developed line in the negative serum group; + indicated a shorter, rounded line in the positive serum group, which may not have appeared until the second day in the negative serum group; ++ and +++ represented intermediate reactions. Observations were also made on the junction of the line or lines of the test serum with those of the positive serum.

Bovine, goat and rabbit sera occasionally produced two and more rarely three precipitin lines. When the *Br. melitensis* antigen was placed in the central basin and the various sera in the peripheral basins, the lines nearest to the antigen basin generally joined to form a hexagon. The second and third lines produced by sera of different species did not unite in all cases; the lines sometimes crossed or if in apposition there was a distinct break

between the ends (Fig. 1b). Serial dilutions of sera caused the disappearance of the second and third lines before that of the line closest to the antigen basin.

*Rabbit sera*

Eighteen rabbits infected with virulent *Br. abortus* were bled 5, 7 and 9 days and 9 weeks after infection. Agglutinins were present in the sera of all rabbits by the fifth day and were maintained throughout the period of observation. Complement-fixing antibodies and precipitins were detected in some of the sera at 7 and 9 days and in all the sera at 9 weeks. There was no correlation between the presence of complement-fixing antibodies and precipitins in individual sera taken 7 and 9 days after infection.

*Goat sera*

Thirty-three goats inoculated with a smooth killed *Br. melitensis* culture in adjuvant (Jones, Thomson & Alton, 1958) developed serum precipitins by the second week, which persisted for the six months' period of observation. Thirty-four goats inoculated with a living avirulent culture of *Br. melitensis* in saline had serum precipitins at two weeks but they persisted in only twelve cases for four months. Similar numbers of non-vaccinated goats and goats inoculated with a rough killed *Br. melitensis* culture in adjuvant did not have precipitins in their sera.

All goats were challenged by the conjunctival route with virulent *Br. melitensis* and were bled and autopsied four weeks after challenge. The results obtained from goats in the control and rough adjuvant groups, whose sera had been negative before challenge, are as follows. Of 18 sera from goats with a generalized infection as revealed by bacteriological examination, 8 had precipitins, whereas 9 had agglutinins greater than 1/20 and 6 had agglutinins from 1/10 to 1/20. Of 31 sera from goats shown to be free from infection, none had precipitins but 5 had agglutinins from 1/10 to 1/20.

*Bovine sera*

1. Sera from 13 cows of two herds known to be free from brucella infection did not react in the precipitin plate test. As these animals had been vaccinated with Strain 19, seven of the sera gave suspicious reactions and one was positive in the agglutination test.

2. Sera from 48 cattle known to be free from brucella infection, unvaccinated and negative to the agglutination test did not produce precipitin lines.

3. Of seven vaccinated cows from a herd with a history of abortions the sera of three were positive to all serological tests, one was positive to

the agglutination test only (1/160) and three were negative to all tests. A culture of *Br. melitensis* was obtained from one of the cows which was positive to all tests.

4. The serum from a cow whose milk contained *Br. melitensis* was positive in the agglutination and precipitin tests but negative in the complement-fixation test.

5. Twenty-five sera were tested from a herd in Kenya, where an aerobic strain of *Br. abortus* was isolated from five of six foetuses examined. Of

**TABLE 1. COMPARISON OF AGGLUTINATION TITRE AND PRECIPITIN REACTION OF BOVINE SERA FROM AN INFECTED HERD IN KENYA**

| Cow. No. | Agglutination titre | | Precipitin reaction |
|---|---|---|---|
| | 9 months before testing | after testing | |
| 73 | 1/80 | 1/80 | +++ |
| 74 | 1/40 | 1/40 | — |
| 75 | — | — | — |
| 76 | 1/80 | 1/80 | ++++ |
| 77 | | 1/160 | ++++ |
| 78 | | 1/20 | — |
| 79 | — | 1/10 | — |
| 80 | — | 1/20 | — |
| 81 | — | 1/20 | ++ |
| 82 | — | 1/10 | — |
| 83 | 1/160 | 1/640 | ++++ |
| 84 | — | 1/40 | +++ |
| 85 | — | — | — |
| 86 | — | 1/640 | ++++ |
| 87 | — | 1/80 | + |
| 88 | — | 1/10 | — |
| 89 | 1/10 | 1/20 | — |
| 90 | — | 1/10 | + |
| 91 | | — | + |
| 92 | — | — | — |
| 93 | — | 1/640 | ++++ |
| 94 | 1/20 | 1/640 | ++++ |
| 95 | | 1/20 | — |
| 96 | — | 1/20 | — |
| 97 | 1/10 | 1/40 | +++ |

the 13 sera which gave positive precipitin reactions (see Table 1), one was negative to the agglutination test, two had titres of 1/10 and 1/20 respectively, and the remainder had titres of 1/40 or over.

6. A comparison of the precipitin reactions and the agglutination titres of 173 sera from an experimental group of cattle is shown in Table 2. These cattle had been used in a vaccination experiment during the period 1949-55. The infection status of each animal was determined at the time of parturition after challenge with virulent *Br. abortus*, but since the completion of the experiment all the animals have mingled. Thus the history of the cattle as recorded from 1949 to 1955 may not represent the condition at the time of testing in 1957. It is observed from Table 2 that all 75 precipitin positive sera were also agglutinin positive, with the exception of one suspicious reactor (1/20 agglutination titre). Of the 98 precipitin negative sera, 15 were agglutinin positive and the remainder were suspicious or negative to the agglutination test.

**TABLE 2. COMPARISON OF AGGLUTINATION TITRE AND PRECIPITIN REACTION OF 173 BOVINE SERA**

| Agglutination titre | Precipitin reaction | Number of sera | Number with previous history of infection |
|---|---|---|---|
| — or 1/10 | — | 54 | 9 |
| 1/20 | — | 28 | 14 |
| | ± | 1 | 0 |
| 1/40 | — | 12 | 5 |
| | + | 4 | 2 |
| 1/80 | — | 2 | 1 |
| | ± to +++ | 14 | 11 |
| 1/160 | — | 1 | 1 |
| | + to ++++ | 8 | 6 |
| > 1/320 | +++ to ++++ | 49 | 44 |

## Discussion

An interesting feature of this study is that a diffusible antigen which was readily demonstrated in cultures of smooth *Br. melitensis* was not obtained from similar preparations of smooth *Br. abortus* or *Br. suis*. The supernatant fluid from a freshly harvested culture of *Br. melitensis* produced a precipitin line, whereas the only preparation of *Br. abortus* which produced a precipitin line was a phenol saline suspension that had been stored for six months. The trichloracetic acid extract of *Br. melitensis* produced three sharp lines in the plate test within 24 hours. The trichloracetic acid

extract of *Br. abortus*, which is a strong antigen in the complement-fixation test and forms a ring in the capillary-tube precipitin test, produced a faint line in the plate precipitin test only after three to five days. The possibility of antigen excess preventing line formation was dismissed in trials by varying distances between antigen and serum basins and by diluting the antigens. Increasing the distance between basins and diluting the reagents always weakened the reaction in the case of the *Br. melitensis* antigen, and no lines appeared with the *Br. abortus* antigen. (Mansi had found 0.4 cm to be the optimum distance between basins for virus antigen-antibody systems and this distance was also the most satisfactory for the brucella system.) It is likely that the antigen is present in the *Br. abortus* cell, as it stimulates antibodies *in vivo*, but it is either present in very small quantities or has not been successfully released by the present methods.[1]

Huston, Huddleson & Hershey (1934) found a precipitating substance " S " which was readily extracted from *Br. melitensis* but not from *Br. abortus* or *Br. suis*. Hershey, Huddleson & Pennel (1935) later showed that the " S " substance was combined with protein in the albuminoid fraction of all three species but in addition was in the free state in extracts of *Br. melitensis*. This finding is in accord with the general observation that all brucella antisera contain " S " precipitins.

Disaggregated antigen was easily made from the *Br. melitensis* antigen by treatment with 80% acetic acid (Miles & Pirie, 1939). But according to Paterson, Pirie & Stableforth (1947), all known methods were unsatisfactory in converting the *Br. abortus* antigen into a water-soluble material of relatively low molecular weight.

A factor in the present study suggesting that the precipitin antigen of *Br. melitensis* is not linked to protein is its relative stability on heating and treatment with acids and alkalis.

The precipitin plate test may be of some value in classifying brucellae, as the demonstration of a diffusible antigen in a saline suspension was correlated with the serological characteristics of *Br. melitensis* cultures.

There was no correlation between the appearance of complement-fixing antibodies and precipitins in the sera of rabbits bled 7 and 9 days after infection. Although they appeared about the same time they did not always appear simultaneously in the same animal. Agglutinins consistently preceded complement-fixing antibodies and precipitins. Similar findings occurred with goat and bovine sera. It would appear that the three antibodies are qualitatively different. The relationship of the three antibody reactions to infection required further investigation. Results from the Kenya herd (Table 1) give some suggestion that when the agglutination titre is high or is increasing a strong precipitin reaction will occur, and

[1] Olitzki & Sulitzeanu (1957) have since reported that sonic extracts of *Br. suis* and *Br. melitensis* produce 5 and 6 lines respectively, with rabbit antisera, using a gel diffusion method which differed somewhat from that described in the present article.

that when a falling agglutination titre is observed the precipitin reaction may be weak or completely absent. This was also observed in the bovine studies summarized in Table 2. In the latter series there was agreement between the precipitin reaction and the agglutination test in 90% of the cases. Disagreement occurred most frequently when the agglutination titre was low (1/40). These observations suggest that the presence of precipitin antibodies in the serum may be more closely related to actual infection in the animal, i.e., when the infection is overcome precipitins disappear more quickly than the agglutinins.

The significance of the three precipitin lines requires further investigation. The line nearest the antigen basin was, with a few exceptions, the strongest and united with the corresponding line of the neighbouring sera of any species tested. The supplementary lines were usually correlated with high agglutinin titre, but correlation with the infection status of the animal has not been possible to date. The supplementary lines of different species did not usually unite, a result which may indicate the presence of independent antibodies in the sera of each species.

## ACKNOWLEDGEMENTS

The authors express their appreciation to Mr J. R. Lawson and Mr D. J. MacKinnon of the Central Veterinary Laboratory, Weybridge; to Dr A. McDiarmid of the Agricultural Research Council Field Station, Compton, Berks., for providing the bovine sera; and to Dr W. Mansi for helpful discussions on the precipitin test.

## RÉSUMÉ

La technique de diffusion des précipitines sur plaque de gélose, mise au point en 1949 par Ouchterlony pour l'étude des réactions antigène viral/anticorps, a été appliquée par les auteurs aux recherches sur les *Brucella*. Ils ont obtenu, à partir de cultures *lisses* (S) de *Br. melitensis* un antigène diffusant dans la gélose et réagissant avec les précipitines des antisérums de *Brucella*, diffusant elles aussi dans la gélose. La réaction se manifeste sous forme de lignes apparaissant dans la gélose, au nombre de 1-3, par un processus encore inexpliqué. Il n'a pas été possible de préparer avec *Br. abortus* et *Br. suis* un antigène diffusible réagissant avec les précipitines du sérum mises en évidence selon cette méthode. Ce test a un certain intérêt comme critère de classement des *Brucella*, car la présence d'un antigène diffusible dans la gélose peut être considéré comme un des caractères sérologiques de *Br. melitensis*.

Les préparations suivantes de *Br. melitensis* sont antigéniques: les cultures fraiches, de deux jours, sur gélose inclinée; le liquide surnageant de suspensions cellulaires en eau physiologique, centrifugées; les extraits de *Brucella* traitées pendant 24 heures par le phénol à 2,5%; l'extrait de *Brucella* traitées pendant 3 heures, au froid, par l'acide trichloracétique à 12,5%. L'antigénicité de suspensions de *Brucella* s'est maintenue malgré le chauffage à 60°C pendant 1 heure; l'ébullition pendant 10 minutes; l'alternance rapide et répétée de la congélation et de la décongélation, entre un bain de neige

carbonique à —76°C et un bain-marie à 37°C; le traitement à l'éther pendant 48 heures à la température ambiante (le surnageant non soluble dans l'éther est resté antigénique); le traitement par NaOH N/20 pendant 72 heures à la température ambiante.

Le test des précipitines sur gélose a été comparé au test des agglutinines et au test de fixation du complément. On n'a pas constaté de relation entre l'apparition des précipitines et celle des anticorps fixateurs du complément chez des lapins saignés 7-9 jours après infection. L'apparition des agglutinines précède toujours celle des anticorps fixateurs du complément et celle des précipitines, chez le lapin, la chèvre et les bovins. Il semble que ces trois groupes d'anticorps diffèrent qualitativement. L'étude des sérums d'un troupeau semble indiquer qu'à un titre élevé en agglutinines correspond une forte réaction des précipitines, et que, lorsque le titre baisse, la réaction est faible ou nulle. Des observations suggèrent en outre que la présence dans le sérum d'anticorps précipitants est en rapport étroit avec l'infection chez l'animal. Lorsque cette dernière est jugulée, les précipitines disparaissent du sérum plus rapidement que les agglutinines.

## REFERENCES

Bjorkland, B. (1952) *Proc. Soc. exp. Biol. (N. Y.)*, **79**, 319
Hershey, A. D., Huddleson, I. F. & Pennell, R. B. (1935) *J. infect. Dis.*, **57**, 183
Huston, R. C., Huddleson, I. F. & Hershey, A. D. (1934) *Techn. Bull. Mich. agric. Exp. Sta.*, No. 137
Jones, L. M., Thomson, P. D. & Alton, G. G. (1958) *J. comp. Path.*, **68** (In press)
Mansi, W. (1957) *J. comp. Path.*, **67**, 297
Miles, A. A. & Pirie, N. W. (1939) *Brit. J. exp. Path.*, **20**, 83
Olitzki, A. & Sulitzeanu, D. (1957) *Bull. Res. Coun. Israel*, **6E**, 112
Paterson, J. S., Pirie, N. W. & Stableforth, A. W. (1947) *Brit. J. exp. Path.*, **28**, 223
Stableforth, A. W. (1954) *Bull. Wld Hlth Org.* **10**, 927
World Health Organization, Expert Committee on Biological Standardization (1954) *Wld Hlth Org. techn. Rep. Ser.*, **86**